CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER: NDA 20405

ENVIRONMENTAL ASSESSMENT AND/OR FONSI

ENVIRONMENTAL ASSESSMENT

AND

FINDING OF NO SIGNIFICANT IMPACT

FOR

LANOXIN TABLETS

(DIGOXIN)

NDA 20-405

FOOD AND DRUG ADMINISTRATION

CENTER FOR DRUG EVALUATION AND RESEARCH

DIVISION OF
CARDIO-RENAL DRUG PRODUCTS
(HFD-110)

FINDING OF NO SIGNIFICANT IMPACT

NDA 20-405

LANOXIN TABLETS

[DIGOXIN]

The National Environmental Policy Act of 1969 (NEPA) requires all Federal agencies to assess the environmental impact of their actions. FDA is required under NEPA to consider the environmental impact of approving certain drug product applications as an integral part of its regulatory process.

The Food and Drug Administration, Center for Drug Evaluation and Research has carefully considered the potential environmental impact of this action and has concluded that this action will not have a significant effect on the quality of the human environment and that an environmental impact statement therefore will not be prepared.

In support of their new drug application for Lanoxin Tablets, GlaxoWellcome conducted a number of environmental studies and prepared an environmental assessment in accordance with 21 CFR 25.31a (b) (5) (attached) which evaluates the potential environmental impacts of the manufacture, use and disposal of the product.

Digoxin is a drug substance obtained from natural sources which is administered as an oral tablet for the treatment of congestive heart failure. The drug substance is isolated and purified by The Wellcome Foundation Ltd., Kent, UK and Burrows Wellcome Co., Greenville, NC. The drug product is manufactured by Burrows Wellcome in Greenville, NC and Kirkland, Quebec, Canada. The finished drug product will be used in hospitals, clinics and by patients in their homes.

The drug substance and its metabolites will be excreted in urine into the sewer system. Chemical and physical test results indicate that they will be restricted to the aquatic environment.

As the drug substance, digoxin, is expected to persist in the aquatic environment for some time, its toxicity to an aquatic organism was determined. Results indicate that the drug substance is generally not toxic to Daphnia magna at concentrations at least 100 times greater than the maximum expected environmental concentration (MEEC).

Disposal includes out of specification lots, returned, unused or expired product, empty or partly used product and packaging. These will be disposed at licensed waste water treatment, incineration facilities and landfills. Empty or partially empty packages generated in American hospitals and clinics will be disposed according to their regulations. Empty or partially empty containers from home use will be disposed in the community solid waste management system which may include landfills, incineration and recycling. Minimal quantities of unused drug may be disposed in the sewer system.

The Center for Drug Evaluation and Research has concluded that the product can be manufactured, used and disposed without any expected adverse environmental effects. Precautions taken at the sites of manufacture of the bulk product and its final formulation are expected to minimize occupational exposures and environmental release.

Adverse effects are not anticipated upon endangered or threatened species or upon property listed in or eligible for listing in the National Register of Historic Places.

12/15/95 Florian Zielinski
DATE PREPARED BY: Florian Zielinski, Review Chemist
Division of New Drug Chemistry I

12/19/95 Robert Wolters
DATE DIVISION CONCURRENCE: Robert J Wolters,
Division of New Drug Chemistry I

1/16/96 Nancy B Sager
DATE CONCURRED: Nancy B. Sager
Environmental Scientist, Center for Drug Evaluation and Research

Attachments: Environmental Assessment
Material Safety Data Sheet (drug substance)

Original: NDA 20-405
HFD-004 FONSI File [NDA # 20-405]
HFD-004 Docket File
HFD-019 FOI COPY
HFD-110 Division File
HFD-110 CSO, Gary Buehler
HFD-110 Review Chemist, Florian Zielinski

BURROUGHS WELLCOME CO.
Greenville, NC, USA

Wellcome

GCPV/95/0005/01

LANOXIN (digoxin) Environmental Assessment Information

B B Franklin
Chemical Development Laboratories, Greenville, NC, USA

S R Alston
Environmental Services, Greenville, NC, USA

17 October 1995

Brenda Bunch Franklin — 18 October 1995
B B Franklin — Date

Sarah R. Alston — 19 October 1995
S R Alston — Date

LANOXIN (digoxin) TABLETS
ENVIRONMENTAL ASSESSMENT INFORMATION

1. DATE:

October 17, 1995

2. APPLICANT/PETITIONER:

Burroughs Wellcome Co.

3. ADDRESS:

3030 Cornwallis Rd.
Research Triangle Park, NC 27709

4. DESCRIPTION OF PROPOSED ACTION

4.a Describe the requested approval

Burroughs Wellcome Co. has filed a New Drug Application for approval to manufacture, package, and market LANOXIN† (digoxin) Tablets, 0.0625 mg digoxin per tablet, 0.125 mg digoxin per tablet, 0.1875 mg digoxin per tablet, 0.25 mg digoxin per tablet, 0.375 mg digoxin per tablet and 0.5 mg digoxin per tablet, for the treatment of heart failure and other cardiac conditions. This application is for Burroughs Wellcome Co. (USA) to isolate and purify digoxin itself as well as manufacture, package and market LANOXIN Tablets. This application is also for The Wellcome Foundation. Ltd. (UK) to purify digoxin, for Burroughs Wellcome, Inc., Kirkland, Quebec, Canada, to formulate a product containing digoxin into tablets and for Burroughs Wellcome Co. (USA) to market the resulting product in the United States as LANOXIN Tablets.

4.b Describe the need for the action

LANOXIN Tablets are indicated for the treatment of heart failure. The drug may be used alone or in combination with diuretics, angiotensin converting enzyme inhibitors, or vasodilators. It is marketed as an injection (I.M. and I.V.), capsules, an elixir and tablets. The estimated patient population is three million people per year.

Digoxin is one of the cardiac (or digitalis) glycosides, a closely related group of drugs having in common specific effects on the myocardium. These drugs are found in a number of plants. Digoxin is extracted from the leaves of *Digitalis lanata*. The term "digitalis" is used to designate the whole group.

† This is a Trade Mark of Wellcome group companies.
Registered in U.S. Patent and Trademark Office.

The glycosides are composed of two portions, a sugar and a cardenolide (hence "glycosides").

Mode (Mechanism) of Action: Digoxin inhibits sodium–potassium ATPase, an enzyme that regulates the quantity of sodium and potassium inside cells. Inhibition of the enzyme leads to an increase in intracellular concentration of sodium and thus (by stimulation of sodium<calcium exchange) an increase in the intracellular concentration of calcium. The beneficial effects of digoxin result from direct actions on cardiac muscle, as well as indirect actions on the cardiovascular system mediated by effects on the autonomic nervous system. The autonomic effects include: 1) a vagomimetic action, which is responsible for the effects on the sinoatrial and atrioventricular nodes; and 2) baroreceptor sensitization, which results in increased afferent inhibitory activity and reduced activity of the sympathetic nervous system and renin–angiotensin system for any given increment in mean arterial pressure. The pharmacologic consequences of these direct and indirect effects are: 1) an increase in the force and velocity of myocardial systolic contraction (positive inotropic action); 2) a decrease in the degree of activation of the sympathetic nervous system and renin–angiotensin system (neurohormonal deactivating effect); and 3) slowing of the heart rate and decreased conduction velocity through the atrioventricular node (vagomimetic effect). The effects of digoxin in heart failure are mediated by its positive inotropic and neurohormonal deactivating effects.

4.c Describe location(s) where the product(s) are to be

(1) Produced

The drug substance, digoxin, will be isolated and purified at Burroughs Wellcome Co., Corner of US 13/NC 11 and Hwy. US 264, Greenville, NC, 27834, USA. Digoxin will also be purified at The Wellcome Foundation Ltd., Temple Hill, Dartford, Kent, DA1 5AH, UK. The drug product will be formulated at Burroughs Wellcome Co., Corner of US 13/NC 11 and Hwy. US 264, Greenville, NC, 27834, USA, and Burroughs Wellcome, Inc., 16751 Trans Canada Highway, Kirkland, Quebec, Canada, H9H 4J4.

(2) Used

LANOXIN Tablets are intended for use in humans with heart failure, atrial fibrillation, atrial flutter, paroxysmal atrial tachycardia (PAT) and atrial arhythmia. Usage of this product will occur in hospitals and homes (out patient care) throughout the United States (USA).

(3) Disposed

The Wellcome Foundation Limited (UK)

Drug product, for the US market, will not be produced at The Wellcome Foundation Limited in the UK. Information on disposal procedures for the drug substance purification process can be found in Section 6.b of this report.

Burroughs Wellcome Co. (USA)

Disposal of expired or rejected drug product will be accomplished through on-site waste management procedures which minimize threats to human health or the environment. Material is destroyed either by shredding and rinsing followed by wastewater treatment, or by incineration.

The shredder facility combines shredding of the material through a Milpac shredder with a water wash of the shredded material. Wastewater from the wash operation is discharged to an on-site biological pretreatment facility and then discharged to the Greenville Utilities Publicly Owned Treatment Works under Pretreatment Permit BW01-98. This permit expires on May 3, 1998. Solids generated during the shredding process are disposed of off-site at the Bertie County Regional Landfill under local County ordinances.

Alternatively, the material may be destroyed by an on-site controlled air incinerator which operates at temperatures ranging from 1200°F in the primary chamber to 1850°F in the secondary chamber. The incinerator operates under N.C. Division of Solid Waste Permit No. 74-03-I. The solid waste incinerator permit is scheduled for review on July 7, 1997 (every five years). Inert materials, such as glass and foil wrap, remain with the ash residue which is managed according to specified permit conditions. Cardboard packaging is destroyed and remains mostly as ash material. Volatile emissions from PVC packaging are released into the secondary chamber and converted to water, carbon dioxide, and hydrogen chloride. The gases are further treated in a flux force collision scrubber which removes particulate and hydrogen chloride contaminants. The incinerator and ancillary equipment (scrubber and waste feed components) have all been designed to meet Federal and State air emission standards for particulates, hydrogen chloride, and metal compounds. Burroughs Wellcome Co.'s Greenville facility has received an AQ-22, Permit No. 5754R52, for air emissions from production and manufacturing operations. This permit expires on January 1, 1996. Information on incinerator emission limits can be found in Section 6.a of this report.

Burroughs Wellcome Inc. (Canada)

The active ingredient, digoxin, will be imported from Burroughs Wellcome Co. (USA), so waste resulting from the production of the drug substance, digoxin, is not a consideration.

Any raw materials, expired, returned or rejected product, or contaminated packaging components, will be disposed of by incineration at a licensed facility with a permit for destruction of pharmaceutical and/or hazardous waste. The contractor handling non-hazardous pharmaceutical waste destined for incineration is Laidlaw. The permit is issued by the Quebec Minister of the Environment, and has certification number 27463446 and reference number 7610-07-01-00008-09. The contractor handling hazardous waste destined for incineration is ChemTech. The permits for transportation

and treatment of hazardous materials are issued by the Quebec Minister of the Environment, with reference numbers G-7610-16-01-0173000 1086915 and 7610-16-01-0453056 1074390, respectively.

4.d **Describe the type of environment present at and adjacent to production and disposal sites.**

The Wellcome Foundation Limited (UK)

The Wellcome Foundation Limited site, where drug substance purification will take place, is located in an industrial area adjacent to the town of Dartford, Kent, UK. Dartford is in the Thames estuary region in a temperate zone.

Burroughs Wellcome Inc. (Canada)

The Canada production site where drug product formulation will take place is located in Kirkland, Quebec, Canada. The municipality of Kirkland is on the Island of Montreal which is situated between the River "Des Prairies", to the north, and the St. Lawrence River, to the south. Beyond the northern boundary is the industrial city of Laval and following the southern boundary is a rural plain dominated by farms and towns which extend to Vermont and upper New York State. The Kirkland site is located in a temperate zone and its climatic region is defined as continental.

Burroughs Wellcome Co. (USA)

The United States production site where drug substance and drug product will take place is located on the outskirts of Greenville, North Carolina. The manufacturing plant is situated in an industrial park surrounded by a mostly rural area. Greenville is in a coastal plain region in a temperate zone.

Treatment and/or disposal of rejected or returned goods will take place both on site at the Burroughs Wellcome Co. wastewater pretreatment plant and solid waste incineration facility, and off site at the Greenville Utilities Commission (GUC) Wastewater Treatment Plant, and the Bertie County Regional Landfill. No special permits or licenses are required for disposal of materials at the Bertie County Regional Landfill. Nonhazardous waste requiring special handling, such as ash and wastewater treatment sludges, are accompanied to the landfill by a waste manifest in accordance with landfill requirements.

The Burroughs Wellcome Co. on-site wastewater pretreatment plant is located in the northeastern section of the facility, within the Environmental Complex. Unit operations consist of first and second stage aeration tanks for biological stabilization, secondary clarifiers for removal of sludge solids, a neutralization tank, surge tanks, and effluent discharge with flow monitoring equipment. The pretreatment facility is designed to handle a maximum sustained daily flow rate of 410,000 gallons. Flexibility to operate within a wide range of organic and hydraulic loadings is provided by a system of surge tanks for

high strength wastewater or hydraulic surges in excess of the design capacity. Effluent from the pretreatment plant is discharged to the GUC wastewater treatment plant. The plant operates under a permit granted by GUC, BW01–98.

The on–site incineration facility is also located in the Environmental Complex and houses a 2,000 lb/h controlled air solid waste incinerator, flux forced collision scrubber, automatic waste feed through a rotary shear shredder, and nearly 2,000 sq. ft. of storage space. The incinerator/scrubber combination have been designed to meet or exceed Federal and State air quality limitations on particulates and chlorides. The facility is operated under permits granted by both the N.C. Division of Solid Waste, Permit No. 74–03–I, and the N.C. Air Quality Management Section, Permit No. 5754R52.

The GUC Wastewater Treatment Plant is situated in an area of mixed industry and rural settings. The treatment plant serves the City of Greenville and contiguous areas. Treated wastewater from the plant is discharged into the Tar River, which empties into the Pamlico Sound. GUC operates the Wastewater Treatment Plant under authority granted by the North Carolina Division of Environmental Management, NPDES Permit No. NC001058.

The Bertie County Regional Landfill is located about an hour north of Greenville on the outskirts of Windsor, NC in a rural area surrounded by farmland and forested land. The capacity of the landfill is approximately 11 million cubic yards divided into four cells to be phased in over twenty years from 1993. The landfill is managed by a private corporation under the auspices of an eight–county government in accordance with the N.C. Department of Environment, Health and Natural Resources Permit No. 08–03.

5. IDENTIFICATION OF CHEMICAL SUBSTANCES THAT ARE SUBJECT TO THE PROPOSED ACTION

5.a Nomenclature

Drug Substance

Generic Name:	digoxin See Attachment I for the Material Safety Data Sheet
Chemical Name [IUPAC]:	3ß–[(O–2,6–Dideoxy–(ß–D–*ribo*–hexopyranosyl–(1–4)–O–2,6–dideoxy–ß–D–*ribo*–hexpyranosyl)–(1–4)–2,6–dideoxy–ß–D–*ribo*–hexopyranosyl)oxyl]– 12ß, 14–dihydroxy–5ß–card–20(22)–enolide
Chemical Name [Chem. Abstr.]:	(3ß, 5ß, 12ß)–3–[(O–2,6–Dideoxy–(ß–D–*ribo*–hexopyranosyl–(1–4)–O–2,6–dideoxy–ß–D–*ribo*–hexpyranosyl)–(1–4)–2,6–dideoxy–ß–D–*ribo*–hexopyranosyl)oxyl]–12ß, 14–dihydroxy–5ß–card–20(22)–enolide

Drug Product

LANOXIN Tablets

See information in CONFIDENTIAL Appendix A.

5.b **CAS Registration Number**

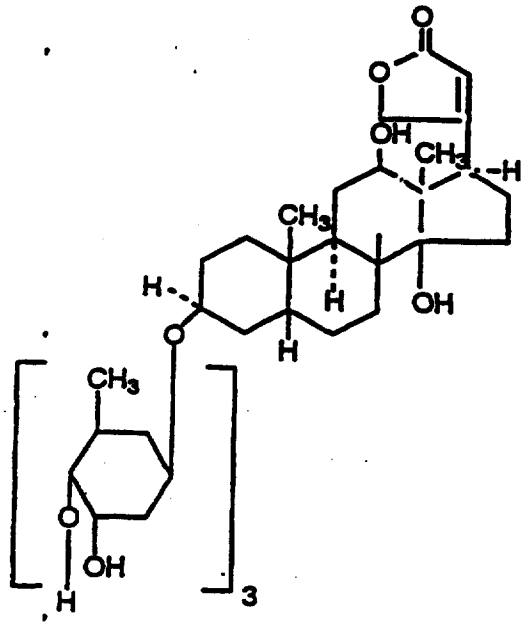
[20830-75-5]

5.c **Molecular Weight and Formula**

$C_{41}H_{64}O_{14}$

MW = 780.95

5.d **Structural Formula**



5.e **Physical Description**

Odorless, clear to white crystals, or white crystalline powder

5.f **Additives and Impurities**

See information in CONFIDENTIAL Appendix A.

6. INTRODUCTION OF SUBSTANCES INTO THE ENVIRONMENT

6.a **For the production site, Burroughs Wellcome Co. (USA)**

(1) **Substances expected to be emitted**

A list of materials used in the manufacture of digoxin is furnished in CONFIDENTIAL Appendix B. The Occupational Exposure Limits and a

discussion of controls exercised within the process designed to prevent or minimize their emission into the environment are also included. Chemical Abstract Service numbers are provided in CONFIDENTIAL Appendix B to facilitate acquisition of Material Safety Data Sheets for OSHA regulated materials.

A list of materials used in the production of LANOXIN Tablets is furnished in CONFIDENTIAL Appendices A and B. The Occupational Exposure Limits and a discussion of controls exercised within the process designed to prevent their emission into the environment are also included. Chemical Abstract Service numbers, when available, are provided in CONFIDENTIAL Appendix B to facilitate acquisition of Material Safety Data Sheets for OSHA regulated materials.

(2) **Controls exercised**

Air Emissions

Air emissions from the manufacture of digoxin are controlled and minimized using condensers on the reactor tanks, dust collectors, and carbon absorption systems installed on the solvent recovery equipment. All of the air emission control devices are covered under Air Permit no. 5754R52. Permit limitations are converted under State Toxic Air Pollutant Standards, and VOC limits. CONFIDENTIAL Appendix B contains specific constituents and permit limits. Standard Operating Procedures are carried out in the manufacturing process to control air emissions generated through nitrogen sweeping of lines and reactor vessels.

No air emissions are expected to occur during formulation of LANOXIN Tablets.

Information on the incineration of solid wastes is included in Section 6.a (2) *Solid Waste.*

Liquid Waste

Flammable liquid waste generated from the manufacture of digoxin is managed through special waste lines to the on-site liquid waste incinerators. The incinerators are operated under a site permit granted by EPA and the State of North Carolina, Permit ID NCD047373766. Procedures for operating the incinerators and waste transfer lines are covered in SOP's titled "Flammable Waste Incinerator Inspection" and "Flammable Waste Tank and Piping Operating Procedures."

Solid Waste

The nonhazardous solid waste is either managed on site through incineration or shredding. The solid waste incinerator is operated under a permit granted by the N.C. Division of Solid Waste, Permit No. 74-03-I. Procedures for

operating the solid waste incinerator are covered in an SOP titled "Operation of the Solid Waste Incinerator." Expired, rejected or returned product and packaging material is physically destroyed in the solid waste incinerator or by an on-site Milpac shredder. All shredded packaging material, glass, and plastic is incinerated on site or may be disposed off site at the Bertie Regional Landfill under local County ordinances. Shredder operations are covered in SOP's titled "Labeling, Disposition, and On-Site Destruction of Product-Related Waste" and "Waste Destruction Area Daily Inspection."

Wastewater

Wastewater generated from production of drug substance is discharged to the Burroughs Wellcome Co. Wastewater Pretreatment Facility. Wastewater generated from formulation of drug product is discharged directly to the Greenville Utilities commission (GUC) Publicly Owned Treatment Works. The pretreatment facility is operated in accordance with Discharge Permit, BW01-98, which permits the discharge of the treated process and pharmaceutical wastewater to the Greenville Utilities Commission (GUC) treatment plant. Procedures for operating the Wastewater Treatment facilities are covered in an SOP titled "Wastewater Pretreatment Plant and Wastewater Monitoring Operations, Inspection/Corrective Actions."

(3) **Citation of and statement of compliance with applicable emission requirements**

Burroughs Wellcome Co. states that it is in compliance with, or on an enforceable schedule to be in compliance with, all emission and occupational control requirements set forth in permits, consent decrees, and administrative orders applicable to the production of digoxin and LANOXIN Tablets, at its facilities in the United States, as well as emission requirements set forth in Federal, State, and local statutes and regulations applicable to the production of digoxin and LANOXIN Tablets, at its facilities in the United States.

All of the following applicable emissions and/or occupational control requirements are complied with on a daily basis for the production of digoxin and LANOXIN Tablets, at Burroughs Wellcome Co.'s Greenville, North Carolina, facility:

Federal

Direct point source discharges from the manufacturing facility are governed by 40 CFR 122 – The National Pollutant Discharge Elimination System (NPDES). Wastewater generated at the manufacturing facility, or during destruction of off-specification or returned product are governed by 40 CFR 403 – EPA Standards, and 40 CRF 439 – Guidelines and Standard for Pharmaceutical Manufacturing. Both the NPDES program and wastewater management programs are authorized by the Federal Water Pollution Control Act, PL 92-500 (also known as the Clean Water Act). The objective of this act is to restore and maintain the chemical, physical and biological integrity of the nation's water.

Air emissions from the facility are governed by the general provisions of 40 CFR 60 – EPA Regulations on Standards and performance for New Stationary Sources. Air Quality Management Programs are authorized by the Clean Air Act, PL 90–128. The purpose of the act is to protect and enhance the quality of the nation's air resources, develop and maintain a program to achieve the prevention and control of air pollution and to encourage reasonable governmental actions for pollution prevention.

Hazardous wastes generated and treated at the manufacturing facility are governed by the general provisions of 40 CFR 260–280 – Hazardous Waste Management System. Hazardous Waste Management Programs are authorized by the Resource Conservation and Recovery Act of 1976, PL 94–580. The objectives of the act are to promote the protection of health and the environment and to conserve valuable material and energy resources.

State

The North Carolina Department of Environment, Health and Natural Resources requires permits covering the operation of air pollution abatement facilities and/or emission sources from manufacturing operations; point source discharges to surface waterways; and the generation and treatment of Hazardous Waste. Burroughs Wellcome Co.'s Greenville facility has received an AQ–22, Permit No. 5754R52, for air emissions from production and manufacturing operations. This permit expires on January 1, 1996. Burroughs Wellcome Co.'s Greenville facility has received a permit, NCD047373766, to operate a hazardous waste treatment and storage facility, and permit No. 74–03–I for operation of a solid waste incinerator. The hazardous waste management permit expires on October 15, 1999. The solid waste incinerator permit is scheduled for review on July 7, 19997 (every five years). Burroughs Wellcome Co. has received an NPDES Discharge Permit, NC001058, for noncontact cooling water point source discharge to Parker's Creek, North Carolina. This permit expires February 28, 2000.

Local

All wastewater from the Burroughs Wellcome Co. manufacturing facility is discharged to the Greenville Utilities Commission's Publicly Owned Treatment Works under a Pretreatment Discharge Permit No. BW01–98. This permit expires on May 3, 1998.

(4) **Effect of the action upon compliance with current emissions requirements**

A five–year production estimate is included as information in CONFIDENTIAL Appendix B.

Waste streams resulting from the manufacture and formulation of LANOXIN Tablets will include solid waste, and wastewater. Each waste stream is described below and its impact on current emissions requirements is addressed.

Air Emissions

Potential air emissions from the manufacture of digoxin (drug substance) will come from chemical manufacturing facility as air toxic constituents and volatile organic compounds (VOC's). Control devices installed in the facility are carbon block condensers and carbon absorption units. Digoxin manufacture has been in place prior to submission of any recent air permit modifications and thus no permit modifications will be required for this process.

No air emissions are expected to occur during formulation of LANOXIN Tablets.

A discussion of potential emissions resulting from incineration of solid wastes can be found in Section 6.a (4) *Solid Waste.*

Solid Waste

Nonhazardous solid waste is generated both from the manufacture of digoxin and formulation of LANOXIN Tablets. Solid waste generated through manufacture of digoxin consists of two bi-products from the manufacturing process. One is spent digitalis leaf from which the active ingredient is extracted and the other is a spent filter media. These two waste streams are disposed off site at a lined landfill located in Bertie County, NC. (See Section 4.d for a description of the landfill facility.)

The solid waste generated from formulation of the drug product (LANOXIN Tablets) is primarily glass, packaging material, returned goods and rejected product. This waste is treated on site either through use of the crusher facility or the solid waste incinerator [see 4.c.(3)]. The amount of rejected product from formulation processes is expected to be kept to a minimum acceptable reject standard, and would therefore not impact the ability of the waste treatment systems to handle the waste. Treatment of the process waste and rejected product through the on-site treatment facilities would consume less than 0.14% per year of the unit's waste handling capacity, based on 1999 (fifth year) production forecasts. Permits applicable to these waste treatment systems are the local Pretreatment Discharge Permit No. BW01-98 for the crusher facility, and N.C. Department of Environment, Health and Natural Resources Air Quality Permit No. 5754R52 and N.C. Division of Solid Waste Permit No. 74-03-I for the solid waste incinerator.

Specific permit limitations controlling incineration of solid waste are VOC limits in Air Quality Permit No. 5754R52. Solid waste generated from formulation of LANOXIN Tablets, will not contain any compounds restricted by these site permits. No impact is expected on the current emissions requirements for solid waste management.

Wastewater

Wastewater will be generated from production processes through equipment cleanup and from waste disposal processes using the crusher facility's

shredding and rinsing procedure [see 6.a(2)]. The only potential area of impact on emissions limitations is the local Pretreatment Discharge Permit No. BW01-98, specifically flow limitations. However, because the equipment to be used for production and disposal processes is currently in place, no increase in flow is anticipated; therefore, no impact on the permit is expected. The projected wastewater generation from production of the drug substance will use up approximately 0.32% of the permitted discharge capacity of the wastewater pretreatment operations.

Modifications which will significantly impact the hydraulic or pollutant loading require notification to the local Publicly Owned Treatment Works (POTW). Depending on the impact, negotiations with the local authority to allow permit modifications may be required. All modifications are reviewed and evaluated in-house by the Environmental Services Department. Formulation of drug product is not expected to trigger notification or modification of the permit.

Liquid Waste

Flammable waste is both recovered during the manufacturing process and treated on site by liquid waste incinerators. The liquid waste incinerators are operated under EPA permit No. NCD047373766 granted by the state of North Carolina. Incineration of flammable waste generated from the digoxin (drug substance) manufacturing process will consume approximately 1.85% of the permitted incineration capacity in 1999 (fifth year of production). Incineration of digoxin hydrochloride production waste streams will not impact current permit limitations.

In the event incinerator capacity is exceeded, Burroughs Wellcome Co. will evaluate additional recovery alternatives or possibly design and construct additional treatment facilities and submit the necessary permit applications.

6.b For the production site – The Wellcome Foundation Limited (UK)

(1) Substances expected to be emitted

A list of materials used in the manufacture of digoxin is furnished in CONFIDENTIAL Appendix B. The Occupational Exposure Limits and a discussion of controls exercised within the process designed to prevent their emission into the environment are also included. Chemical Abstract Service (CAS) numbers, when available, are provided in CONFIDENTIAL Appendix B to facilitate acquisition of Material Safety Data Sheets for OSHA regulated materials.

(2) Controls exercised

Air Emissions

Gaseous emissions are under the control of Her Majesty's Inspectorate of Pollution, Integrated Pollution Control in Part 1 of the Environmental Protection Act 1990. The working environment is also controlled in accordance with the Health and Safety Executive Occupational Exposure Limits EH40 which is regularly updated.

Liquid Wastes

Liquid wastes containing organic solvents are either recovered and re-used in the process, incinerated on site in a facility authorized by Her Majesty's Inspectorate of Pollution, Authorization No. AG9264 (Environmental Protection Act 1990), or directed off site to an appropriate high temperature facility authorized by Her Majesty's Inspectorate of Pollution. Authorizations granted by Her Majesty's Inspectorate of Pollution will not expire but will be reviewed on a regular basis (every 4 years). Alternatively, material will be directed off site for disposal by a licensed contractor with agreement from the appropriate Waste Regulation Authority. Where appropriate, waste may be directed to a commercial operator for the recovery of reclaimable constituents.

Solid Waste

General factory waste, including packaging materials, is either incinerated on site in a facility authorized by Her Majesty's Inspectorate of Pollution, Authorization No. AG9264 (Environmental Protection Act 1990) or is directed off site to a landfill site licensed by the Waste Regulation Authority or to incineration facility authorized by Her Majesty's Inspectorate of Pollution. Authorizations granted by Her Majesty's Inspectorate of Pollution will not expire but will be reviewed on a regular basis (every 4 years).

Wastewater

Wastewater is treated at the Wellcome Foundation Limited's effluent treatment plant and then discharged to the sewers under the consent of the local water company, Thames Water Utilities, Consent License Number LS73008A (Water Industry Act 1991). There is no expiration date for this license. The discharge is also authorized by Her Majesty's Inspectorate of Pollution, Authorization No. AK 6853. Authorizations granted by Her Majesty's Inspectorate of Pollution will not expire but will be reviewed on a regular basis (every 4 years).

(3) Citation of and statement of compliance with applicable emission requirements

The Wellcome Foundation Limited states that it is in compliance with, or on an enforceable schedule to be in compliance with, all emission and occupational control requirements in authorizations, consents and regulations applicable to the manufacture of the drug substance, digoxin, at its facilities at Dartford, Kent, the United Kingdom.

(4) **Effect of the action upon compliance with current emissions requirements**

The Wellcome Foundation Limited is licensed to discharge effluent waste to the sewer by the Thames Water Utilities, to discharge cooling water to controlled water by the National Rivers Authority, and to operate processes by Her Majesty's Inspectorate of Pollution. Waste streams resulting from the production of digoxin will include air emissions, solid waste, liquid solvent waste and wastewater.

Air Emissions

Waste process gases from the production of digoxin are not expected to have an adverse impact on emission compliance for the manufacture or incineration process.

Liquid Waste

Liquid waste containing solvent will be directed to the on-site liquid incinerator (Authorization No. AG9264) or will be directed off site to an appropriately licensed high temperature incineration facility, and is not expected to have an adverse impact on the current compliance situation.

Solid Waste

General industrial waste (non-hazardous) will be either incinerated on site in a solid incinerator (Authorization No. AG9264) or will be directed off site to an appropriately licensed landfill site or incineration facility. Rejected process materials will be directed to an appropriately licensed landfill site or incineration facility. These wastes will contribute a small volume to that already dealt with and production is not expected to have an adverse impact on the current compliance situation.

Wastewater

Trade effluent (wastewater) is discharged to the sewer under a license granted by the Thames Water Utilities, consent License Number LS73008A (Water Industry Act 1991). The manufacture of digoxin will result in small volumes of water from cleaning operations and is not expected to have an adverse impact on the current compliance situation.

6.c For the production site, Burroughs Wellcome Inc. (Canada)

(1) Substances expected to be emitted

A list of materials used in the production of LANOXIN Tablets is furnished in CONFIDENTIAL Appendix B. The Occupational Exposure Limits and a discussion of controls exercised within the process designed to prevent their emission into the environment are also included. Chemical Abstract Service

numbers, when available, are provided in CONFIDENTIAL Appendix B to facilitate acquisition of Material Safety Data Sheets for OSHA regulated materials.

(2) **Controls exercised**

Air Emissions

No air emissions are expected to occur during production of LANOXIN Tablets.

Solid Waste

Since digoxin will not be manufactured at this facility, the only solid waste expected is general factory waste, packaging material, expired, returned or rejected product and dust collected by the dust collection system.

General factory waste and uncontaminated packaging material is collected in the on-site compactor and is disposed of at a landfill. Information on the landfill can be found in Section 6.c (4) *Solid Waste.*

Expired, returned or rejected product, contaminated packaging components and dust collected by the dust collection system are incinerated at a facility with a permit for destruction of pharmaceutical and/or hazardous waste. Information on the incineration facilities can be found in Section 6.c (4) *Solid Waste.*

Wastewater

Since digoxin will not be manufactured at this facility, the only wastewater expected is from cleaning operations. Wastewater from cleaning operations is discharged to the sewer under permit number 487 issued by the "Communauté Urbaine de Montréal, Service de l'Environnement."

(3) **Citation of and statement of compliance with applicable emission requirements**

Burroughs Wellcome Inc. states that it is in compliance with, or on an enforceable schedule to be in compliance with, all emission and occupational control requirements set forth in permits, consent decrees and administrative orders applicable to the packaging and labeling of LANOXIN Tablets (drug product), at its facilities in Canada, as well as emission requirements set forth in Federal, Provincial, and local statutes and regulations applicable to the packaging and labeling of LANOXIN Tablets, at its facilities in Canada.

(4) **Effect of the action upon compliance with current emissions requirements**

Waste streams resulting from the production of LANOXIN Tablets will include solid waste, and wastewater. Each waste stream is described below and its impact on current emissions requirements is addressed.

Air Emissions

No air emissions are expected to occur during formulation of LANOXIN Tablets.

Solid Waste

The contractor handling general factory waste and uncontaminated packaging material destined for landfill is WMI du Québec. WMI sends the waste to either the Lachenaie landfill site (authorization no. ES-06-20) or to the Miron landfill site (authorization no. 7523-04).

The contractor handling non-hazardous pharmaceutical waste destined for incineration is Laidlaw. Laidlaw has a permit allowing the destruction of biomedical, pharmaceutical and cosmetic waste. The permit is issued by the Quebec Minister of the Environment, and has certification number 27463446 and reference number 7610-07-01-00008-09. This permit confirms conformance with article 55 of the "Loi sur la qualité de l'environnement (L.R.Q., Chapter Q-2).

The contractor handling hazardous waste destined for incineration is ChemTech. ChemTech has permits allowing for the sampling, analysis, packaging, transportation and treatment of hazardous liquid and industrial wastes. The permits for transportation and treatment of hazardous materials are issued by the Quebec Minister of the Environment, with reference numbers G-7610-16-01-0173000 1086915 and 7610-16-01-0453056 1074390, respectively. The latter permit confirms conformance to article 54 of the "Loi sur la qualité de l'environnement (L.R.Q., Chapter Q-2).

Wastewater

Wastewater resulting from cleaning operations is discharged to the sewer under permit number 487 issued by the "Communauté Urbaine de Montréal, Service de l'Environnement." This permit confirms conformance to regulation 87 issued by the Communauté Urbaine de Montréal. There is no expiration date for this license. The small volume of water used for cleaning operations is not expected to have an adverse impact on the current compliance situation.

6.d **Through use of calculations and/or direct measures, estimate to the extent possible quantities and concentrations of substances expected to enter the environment as a result of use and/or disposal of products affected by the action.**

Calculation of the Maximum Expected Emitted Concentration (MEEC) for the aquatic compartment is included as CONFIDENTIAL information in Appendix B.

The Wellcome Foundation Limited (UK)

In a worst case condition, one assumes that one batch of digoxin could be lost during purification. Such an incident would result in a maximum discharge of 3.2 kg of digoxin. The spilled material would be captured in the Wellcome effluent (136,000 liters/day), resulting in a concentration of 23.54 ppm. This effluent is discharged to the Thames Water Utility treatment plant (205 million liters/day), hence a concentration of 0.0156 ppm. Assuming an 85% efficiency of the sewage treatment plant, the concentration after treatment would be 2.34 ppb. The treatment plant discharges to the Thames River (9092 million liters at the discharge point); therefore, the maximum concentration of digoxin in the Thames River would be 0.051 ppb.

Burroughs Wellcome Co. (USA)

In a worst case situation, assume that one batch of digoxin could be conceivably lost during production. Such an incident would result in a maximum discharge of 1.6 kg of digoxin from its Greenville facility. The permitted daily discharge of wastewater from Burroughs Wellcome Co.'s Greenville facility to the Greenville Utilities Commission is 1.2 million gallons (4.54 million liters) per day. If the total amount were discharged in one day without pretreatment at Burroughs Wellcome Co., this would represent a maximum product concentration of 0.35 ppm discharged to the Greenville Utilities Commission. The maximum daily discharge of wastewater at the Greenville Utilities Commission is 10.5 million gallons (39.7 million liters) per day. The efficiency of the Greenville Utilities Commission biological treatment plant has been measured to exceed 85%. At a worst case of 85%, after treatment, the concentration of the discharge to the Tar River is calculated to be 0.006 ppm. The annual average 7Q10 flow of the Tar River is 101 million gallons (382 million liters) per day. At the Greenville Utility Commission's maximum daily flow rate, the maximum estimated concentration of digoxin in the Tar River would be 0.62 ppb.

Burroughs Wellcome Inc. (Canada)

In a worst case situation, assume that one batch of LANOXIN Tablets could be conceivably lost during production. Such an incident would result in a maximum discharge of 1.2 kg of digoxin from its Kirkland facility. The permitted daily discharge of wastewater from Burroughs Wellcome Inc.'s facility to the municipal sewage treatment facility is 68 thousand gallons (310 thousand liters) per day. If the total amount were discharged in one day without pretreatment at Burroughs Wellcome Inc., this would represent a maximum product concentration of 3.87 ppm discharged to the municipal sewage treatment facility. The maximum daily discharge of wastewater at the municipal sewage treatment facility is 6.3 million gallons (28.8 million liters) per day. The efficiency of the municipal sewage treatment facility biological treatment plant has been measured to exceed 85%. At a worst case of 85%, after treatment, the concentration of the discharge to the St. Lawrence River is calculated to be 0.0062 ppm. The annual average 7Q10 flow of the

St. Lawrence River is 2,708 million gallons (12,312 million liters) per day. At the municipal sewage treatment facility's maximum daily flow rate, the maximum estimated concentration of digoxin in the River Des Prairies would be 0.015 ppb.

7. FATE OF EMITTED SUBSTANCES IN THE ENVIRONMENT

7.a Bioavailability and Metabolites

The bioavailability of digoxin ranges from about 60% to 80%. Following oral administration, a 6 to 8 hour distribution phase is observed. This is followed by a much more gradual serum concentration decline which is dependent on digoxin elimination from the body. Elimination of digoxin follows first-order kinetics; that is, the quantity of digoxin eliminated at any time is proportional to the total body content. Following intravenous administration to normal subjects, 50% to 70% of digoxin dose is excreted unchanged in the urine. Renal excretion of digoxin is proportional to glomerular filtration rate and is largely independent of urine flow. In subjects with normal renal function, digoxin has a half-life of 1.5 to 2.0 days. The half-life in anuric patients is prolonged to 4 to 6 days. Digoxin is not effectively removed from the body by dialysis, exchange transfusion or during cardiopulmonary by-pass because most of the drug is in tissue rather than circulating in the blood.

7.b Aquatic Compartment

In order to assess the effect upon the aquatic compartment, the water solubility, dissociation constant and hydrolysis rate of digoxin were measured.

Water Solubility

The water solubility of digoxin in distilled water is 3.75×10^{-4} M which is greater than 10^{-4} M. The aqueous solubility was not determined at pH 5.0, 7.0, or 9.0, because digoxin is not ionizable.

Hydrolysis Rate

At 37 °C, digoxin does not hydrolyze at pH 7 after 48 hours as reported in the literature (17); therefore, no additional testing was done.

Dissociation Constant

Digoxin has no ionizable functional groups; therefore, no proton dissociation occurs and no dissociation constant can be determined.

Summary

Digoxin can be expected to affect the aquatic compartment based upon the water solubility value.

7.c Air Compartment

In order to assess the effect upon the atmospheric compartment, the vapor pressure of digoxin was estimated, the UV spectra was measured, and the photodegradation evaluated.

Vapor Pressure Estimate

The vapor pressure for digoxin is estimated to be 8.08×10^{-20} torr. The vapor pressure estimate is less than 10^{-7} torr.

UV Spectra

The UV/VIS spectrum of digoxin in distilled water show that, dependent on the solvent or solution studied, digoxin is a UV absorber between 219 and 224 nm.

Photodegradation

Digoxin was one of three cardiac glycosides selected for photostability studies (18). The irradiation was carried out using a rotating reactor (Gräntzel/Karlsruhe), with a low-pressure mercury-lamp (254 nm) or day-light source. An irradiation chamber (Heraeus/Hanau) with a high-pressure mercury-lamp (Q 300) was used for on-plate and petri dish methods. The irradiation was carried out for 96 hours, taking samples intermittently from the mixture every 24 hours.

The percent of digoxin photoproducts increased over the 96 hour period. For the low-pressure mercury lamp test, the amount of photoproducts ranged from 3.2 to 27.8%. For the high-pressure mercury lamp, this amount ranged from 3.8 to 11.1%. For the daylight source, the amount of photoproducts ranged from 0.0 to 1.6%. The greatest degradation was seen with irradiation on a plate where 36.8% photoproducts were observed.

Digoxin showed two main products on irradiation. Based on IR-, ^{1}H NMR- and ^{13}C NMR spectral data, the first product would appear to have a water group cleaved from the sugar moiety. The second product would appear to have the α, β-unsaturated lactone ring of the cardenolide cleaved.

Summary

Based on the estimated vapor pressure, digoxin should not be volatile and would not affect the atmospheric compartment. It is not expected to photodegrade.

7.d Terrestrial Compartment

In order to assess the effect upon the terrestrial compartment, the 1-octanol/water partition coefficient of digoxin was measured.

<u>1-Octanol/Water partition coefficient (log P)</u>
The 1-octanol/water partition coefficient is 1.42.

Summary

The log K_{ow} or log P value is less than 2; therefore, digoxin is not expected to significantly bioconcentrate or to sorb onto organic particles. The terrestrial compartment should not be affected.

8. ENVIRONMENT EFFECTS OF RELEASED SUBSTANCE

Digoxin will most likely affect the aquatic compartment. In order to determine the environmental effects of digoxin on organisms in the aquatic environment, the acute effects on *Daphnia magna* were determined.

Daphnia magna Toxicity

Digoxin was one of 50 reference chemicals whose toxicity was evaluated by determining immobility in *Daphnia magna* during a 24 hour incubation (19). Five concentrations were used for the determination of the EC_{50}. The reported value was calculated using regression analysis after linearization of the dose/response curves by logarithmic transformation of the concentrations. For digoxin, the reported EC_{50} value was 0.031 ± 0.001 mM or 24.2 ± 0.781 mg/mL (ppm).

FDA 1/4/96

Summary

The Calculated EEC (see information in CONFIDENTIAL Appendix B) in the aquatic compartment is compared to 1% of 24.2 ppm (0.242 ppm), the lowest measure of toxicity for digoxin. The calculated EEC is much lower than 0.242 ppm, indicating non-persistence of digoxin in the aquatic compartment. No further testing should be required.

9. USE OF RESOURCES AND ENERGY

9.a Use of Water and Energy

The Wellcome Foundation Limited (UK)

In 1987/88, a combined heat and power plant was established on the Dartford site. Turbines convert gas (or oil) into electricity while waste hot gases raise steam which in turn is used directly for heating or indirectly to operate chillers. Two alternators have a combined output of 12,100 kw and are linked to a high voltage distribution system; any surplus electricity is exported to the National Grid. The combined heat and power plant supports the Company's policies on energy conservation and environmental management. Energy use associated with digoxin production for all formulations will consume less than 1% of the Primary Manufacturing Division's annual production consumption at Dartford.

Water use is estimated at less than 0.1% of annual total plant use. Raw water is supplied from Thames Water Utilities.

Burroughs Wellcome Co. (USA)

Drug Substance

Water used during manufacture of the drug substance is supplied from the Greenville Utilities Commission water treatment plant, which draws its raw water supply from the Tar River. The amount of water used during production is less than 0.5% of total plant use per year. Annual energy use is approximately 1.3% of total plant consumption.

Drug Product

Water used during product formulation, including disposal, is supplied from the Greenville Utilities Commission water treatment plant, which draws its raw water supply from the Tar River. The amount of water used during formulation is less than 0.0002% of total plant use per year. Energy use will constitute less than 0.06% of total plant consumption.

Waste Disposal

Water used in disposal processes during production and formulation will require less than 0.5% of total plant use. The amount of water used will make up less than 0.5% of the permitted daily discharge.

Transportation of Drug Product

Burroughs Wellcome co. will use commercial carriers to transport the drug product from the production site.

Burroughs Wellcome Inc. (Canada)

Drug Product

Water used during product formulation, including disposal, is supplied from the Utilities Commission water treatment plant, which draws its raw water supply from the River. The amount of water used per batch during formulation is less than 0.0001% of total plant use per year. Energy use will constitute less than 0.0006% of total plant consumption.

Waste Disposal

Water used per batch during cleaning processes will require less than 0.006% of total plant use. The amount of water used will make up less than 1.3% of the permitted daily discharge.

9.b **Effects on Endangered or Threatened Species and upon Property Listed in or Eligible for Listing in the National Register of Historic Places**

The Wellcome Foundation Limited (UK):

The Dartford site of the Wellcome Foundation Limited complies with all environmental permits. No endangered or threatened species are affected by

environmental emissions from the drug production. No properties listed with respect to the Listed Buildings and Conservation Area Act 1991, are affected by environmental emissions from the drug production.

Burroughs Wellcome Co. (USA):

The National Historic Preservation Act of 1966 and the Endangered Species Act have been considered prior to issuance of Federal and State environmental permits. No special permit conditions are required as the permitted activity was judged not likely to jeopardize critical habitat nor would the activity impact property listed in or eligible for listing in the National Register of Historic Places. The applicable permits are described in section 6.a(3) for the Greenville, NC (USA) facility.

Approval of the action proposed in this document will not require reevaluation of site environmental permits for their impact on either of these Federal Laws on land management.

Digoxin is prepared by extracting the leaves of the Digitalis lanata. This plant is grown on farms in the Netherlands and is not listed as an endangered species. Burroughs Wellcome Co. uses farm cultures of this plant for the synthesis of digoxin. Details concerning the use of this plant in the synthesis process can be found in CONFIDENTIAL Appendix B.

Burroughs Wellcome Inc. (Canada):

The Kirkland site of Burroughs Wellcome Inc. complies with all environmental permits. No endangered or threatened species are affected by environmental emissions from the drug production.

10. MITIGATION MEASURES

Throughout the development of the synthetic route, consideration was given to the potential environmental impact of the materials used in the process. The thermochemistry, reaction kinetics and the identification and quantification of volatile by–products have been thoroughly considered. The choice and quantities of reagents have been optimized and minimized whenever possible to use the least hazardous materials (from health, safety and environmental considerations) that will complete the desired reaction. Where possible, the use of common reaction media have permitted the use of damp intermediate, thus obviating drying operations.

Burroughs Wellcome Co., Burroughs Wellcome Inc. and The Wellcome Foundation Limited will use existing facilities on site or off site to ensure that releases from this proposed action will have a minimal environmental impact. No special measures outside current Standard Operating Procedures are anticipated or believed required.

The Wellcome Foundation Limited (UK):

The manufacture of drug substance is carried out in equipment and buildings designed for chemical processing. All buildings have drains connected to the

effluent plant. Any digoxin which is accidentally spilled on site will be captured as effluent or by absorbing agents and treated as liquid or solid waste respectively, see Section 6.b(2). Liquid organic solvent waste is disposed via incineration either on site or by a licensed waste disposal contractor. Caustic gas scrubbers are connected to the manufacturing equipment to absorb gaseous by–products. Aqueous waste is treated in the effluent treatment plant, prior to discharge. Solid waste is disposed to landfill by a licensed waste disposal contractor or is directed to an appropriate high temperature incineration facility as described under Section 6.b(2).

Burroughs Wellcome Inc. (Canada):

The manufacture of drug substance is carried out in equipment and buildings designed for chemical processing. All buildings have drains connected to the municipal sewage treatment facility. Any digoxin which is accidentally spilled on site will be captured as effluent or by absorbing agents and treated as liquid or solid waste respectively, see Section 6.c(2). Liquid waste is disposed via incineration by a licensed waste disposal contractor. Solid waste is disposed to landfill by a licensed waste disposal contractor or is directed to an appropriate high temperature incineration facility as described in Section 6.c(2).

Burroughs Wellcome Co. (USA):

Burroughs Wellcome Co. will implement appropriate procedures established for response to spills or releases in the product formulation areas, and in the unlikely event of a release to the environment. The procedures developed to control such releases as well as corporate standards for waste minimization and recycling are described below. These procedures and standards are listed according to the specific area where control is appropriate. The areas relevant to formulation of LANOXIN Tablets are the Primary Manufacturing Division, Solid Dosage Formulations Manufacturing Division and the Environmental Services Department.

10.a **Primary Manufacturing Division (PMD)–Burroughs Wellcome Co. USA**

Manufacture of the drug substance will take place in the Primary Manufacturing Division (PMD) of Burroughs Wellcome Co., USA. Following is a list of PMD SOP's by title, and a brief description:

Procedure for Off–Site Chemical Waste Disposal.

This procedure provides guidance on chemical waste disposal in accordance with Federal and State regulations.

Training Documentation of PMD Facility Personnel in the Handling of Chemical Waste

This SOP provides training procedures for PMD facility personnel in the handling of waste generated within the division to prevent harm to human health or the environment.

Determination of the Composition of PMD Waste Streams

This document provides formal procedures for determining the composition of PMD waste streams.

Reporting of PMD Incidents or Accidents

This procedure outlines the correct reporting actions that should be followed with respect to an incident or accident.

PMD Emergency Plan

This procedure establishes a plan for emergency action within the PMD area, that is consistent with overall plant emergency planning.

PMD Spill Containment Plan

This procedure presents a spill containment plan to assure the rapid and effective mobilization of available resources to contain spills when they occur in the PMD area.

Vent Spill Containment System for PMD Module Building

This SOP describes the Vent Spill Containment System and the operational requirements.

Transfer Lines Between PMD and Environmental Services

This SOP defines the process of flammable waste transfer and identifies through which of the four transfer lines flammable waste will be transferred between PMD and Environmental Services.

Draining Containment Dikes and Trenches in PMD

This document establishes procedures for determining how material in a containment dike or other containment area should be handled. The procedures include criteria for determining whether or not material is contaminated.

Emission Control for Reactors, Crystallizers, and Centrifuges

This procedure was developed to control air emissions from reactors, crystallizers, and centrifuges.

Fugitive Emissions Monitoring

This procedure was developed to ensure that the Organic Vapor Analyzer (OVA) unit is calibrated properly.

Recycling of Fiber Lever-Paks

This document provides procedures for reusing fiber pak drums.

10.b Product Formulation Areas (SDFMD)–Burroughs Wellcome Co. USA

Formulation of the product will take place in the Solid Dosage Formulations Manufacturing Department (SDFMD) of Burroughs Wellcome Co., USA. Following is a list of SOP's by title, and a brief description, for SDFMD:

Dress Code and Safety Precautions for SDFMD

These procedures cover quality and safety procedures for granulating, compressing, coating, and encapsulation areas. Instructions are provided for containing all clothes coverings that may have come in contact with product.

Procedure to Ensure CGMP Compliance in SDFMD

This procedure covers the training plan for properly training employees on Current Good Manufacturing Practices contained in 21 CFR Parts 210 and 211.

Response to Audible Alarms in the Solid Dose Formulations Manufacturing Department

Ensures correct action and responsibilities when the SDFMD Audible Alarms are set off. The procedure covers steps to be taken should an alarm sound indicating a potential problem in the vault where product is held.

Removal of Product Waste from the SDFMD

This SOP covers management of product waste in accordance with Current Good Manufacturing Practices including labeling and disposition of product waste.

Volatile Organic Compound (VOC) Emission Reporting for SDFMD Coating

This procedure provides instruction for properly recording and reporting VOC emissions from SDFMD coating.

10.c Environmental Services Department–Burroughs Wellcome Co. USA

The Environmental Services Department at Burroughs Wellcome Co., USA, is responsible for management of releases to the environment which would pose a threat to human health or the environment. Procedures have been established for notification, response, reporting requirements, and treatment and/or disposal of any contaminated material resulting from a release. Training is provided for all Environmental Services Personnel on waste management and spill prevention and control on a yearly basis. In addition,

all employees in the Environmental Services Department are members of the Hazardous Materials Response Team (HAZMAT) (see item 3, below). HAZMAT is a company wide organization made up of volunteer members from different areas of the plant.

At the plant, releases reaching the stormwater/cooling water collection system are prevented from going off site by a series of holding tanks and spill control gates which automatically close off the stormwater/cooling water ditches if a spill is detected by one of the Astro Units (see item 4, below). Finally, a pumping system may be activated to pump all water out of the stormwater/cooling water ditches and into a 200,000 gallon holding tank.

The SOP's described below cover control of releases to the environment.

Spill Control Contingency Plan (SCCP)

This plan outlines a specific, organized, coordinated course of action for response to fires, explosions, or sudden unexpected release of hazardous waste, non-hazardous waste, or hazardous materials to air, soil, or surface water at the Greenville facility.

Procedure for Monitoring Cooling Water Discharged to Parkers Creek in Compliance with NPDES (National Pollutant Discharge Elimination System) Permit NC001058

This document describes the equipment and procedure for sampling water discharged from the East and West cooling water/rainwater runoff ditches.

Responsibilities of Burroughs Wellcome Co. Hazardous Materials Response Team (HAZMAT)

This SOP outlines the organizational structure, training requirements, equipment and other requirements of the company's Hazardous Materials Response Team (HAZMAT). The HAZMAT Team was organized to operate within the requirements of OSHA Hazardous Waste Operations and Emergency Response Standard (20 CFR 1910.120).

Astro Unit Operating Procedures

Provides operating instructions, guidelines, and inspection procedures for the Astro TOC (total organic carbon)/pH detection alarm units to ensure their reliability and availability at all times for monitoring and recording TOC and pH in the East and West Cooling Water ditches and Barometric sewer line for detection of spills and alarm notification.

Wastewater Pre-treatment Plant (WWPP) and Wastewater Monitoring Operations Inspection/Corrective Actions

This is a procedure to ensure that all systems are functioning properly or that appropriate actions are taken. This SOP outlines the duties of the

Environmental Control Operator relating to the daily inspections conducted in the area of wastewater pre–treatment and monitoring operations.

Labeling, Disposition, and On site Destruction of Product–Related Waste

This procedure details the operating and record keeping requirements which will help to ensure proper labeling, transfer, and disposal of certain wastes containing product, raw or intermediate (e.g., bulk or in–process) pharmaceutical materials. It is primarily intended for waste disposal via crusher, biological treatment and/or off–site disposal of pharmaceutical products and packaging–type materials generated by various plant processes.

Operation of the Solid Waste Incinerator

This procedure details responsibilities and operating parameters for the solid waste incinerator facility. The document covers inspection, start–up and operation of the incinerator and air pollution control equipment, and also for the automatic waste feed conveyor system.

In addition to procedures developed for the manufacturing areas and environmental control are corporate standards for waste minimization and recycling. Specifically:

Corporate Policy #306, Waste Minimization and Disposal

Directs research, development, and production personnel to minimize the quantity and toxicity of industrial wastes generated by the Company, and further, to recycle, reclaim, and reused wastes once they are generated, where economically and environmentally practicable. The policy provides direction for research and development to routinely review manufacturing processes for opportunities to substitute new materials or technologies that might further reduce waste generation or detoxify waste products. The policy commits the company to consider reuse of chemicals, by–products, or products in other research or process operations, and to evaluate spent materials or products for recycling as raw materials in other processes. Finally, the policy directs the Company to consider the possibility of recycling prior to any final destruction or disposal of wastes.

Corporate Policy #307, Solid Waste Recycling

Directs all operating units to establish a goal of minimizing the quantity of solid waste generated by the Company. Options which must be considered are recycling, reclamation and reuse where economically and environmentally practicable. The Company authorizes establishment of site recycling committees charged with the responsibility of developing and implementing comprehensive and solid waste management programs designed to achieve the objectives of this policy.

11. ALTERNATIVES TO THE PROPOSED ACTION

The environmental impact of manufacturing LANOXIN Tablets, is expected to be minimal based on the low toxicity of digoxin in microbial systems, and the low emissions to the environment, where non-persistence was demonstrated in the targeted aquatic compartment. The only alternative to avoid any potential adverse environmental impact is to eliminate the production of this product. However, there would be a cost involved in taking this "no action", namely, that Burroughs Wellcome Co. would not be providing an efficacious and safe drug to patients.

In view of digoxin's low toxicity in aquatic systems and the low expected concentration in the environment (EEC), no adverse environmental effects are expected.

12. LIST PREPARERS OF THE ASSESSMENT (Curricula Vitae are included in ATTACHMENT IX)

NAME	EXPERTISE	EXPERIENCE	PROFESSIONAL DISCIPLINE
Brenda Bunch Franklin	Environmental Assessment	13 years	Environmental Chemistry
Mary F. S. Whisler	Physical Pharmacy	14 years	Physical Pharmacy
Sarah R. Alston	Environmental Engineering	11 years	Engineering
Russell Thompson	Environmental Management	17 years	Environmental Waste Management
Brinda Isings	Environmental Engineering	10 years	Engineering

13. CERTIFICATION

The undersigned representative of Burroughs Wellcome Company certifies that the information presented is true, accurate, and complete to the best of the knowledge of the firm or agency responsible for preparation of this environmental information.

Date: 18 October 1995

Signature of responsible official: Richard M. Rodebaugh

Name (typed): Richard M. Rodebaugh, Ph.D.

Title: Department Head, Chemical Development Laboratories

Date: October 19, 1995

Signature of responsible official: Michael L. Esarey

Name (typed): Michael L. Esarey

Title: Director, Environmental Services

14. REFERENCES

1. *Interim Guidance to the Pharmaceutical Industry for Environmental Assessment Compliance Requirements for the FDA.* Vol. 7, Pharmaceutical Manufacturers Association, 1991.

2. "Technical Assistant Document (TAD)," *FDA Environmental Assessment Technical Assistance Handbook*, NTIS PB87–175345."

3. *Handbook of Chemical Property Estimation Methods*, W.J. Lyman, W.F. Reehl, and D.H. Rosenblatt, American Chemical Society, Washington D.C., 1990.

4. *FDA NEPA Strategy Perspective*, P. G. Vincent Ph.D., Invitational Paper Presentation, American Institute of Chemical Engineers, Pittsburgh, PA, August 18–21, 1991.

5. U.S. Environmental Protection Agency, CFR Part 40 § 122, The National Pollutant Discharge Elimination System (NPDES).

6. U.S. Environmental Protection Agency, CFR Part 40 § 403, EPA Standards

7. U.S. Environmental Protection Agency, CFR Part 40 § 439, Guidelines and Standards for Pharmaceutical Manufacturing

8. U.S. Environmental Protection Agency, CFR Part 40 § 60, EPA Regulations on Standards and Performance for New Stationary Sources

9. U.S. Environmental Protection Agency, CFR Part 40 § 260–280 – Hazardous Waste Management System.

10. Federal Water Pollution Control Act, PL 92–500

11. Clean Air Act PL 90–128.

12. Clean Water Act PL 95–217, as amended.

13. Resources Conservation and Recovery Act (RCRA) PL 94–580 of 1976, as amended.

14. Environmental Protection Act, Parts I & II, United Kingdom.

15. Water Resources Act, United Kingdom, 1991.

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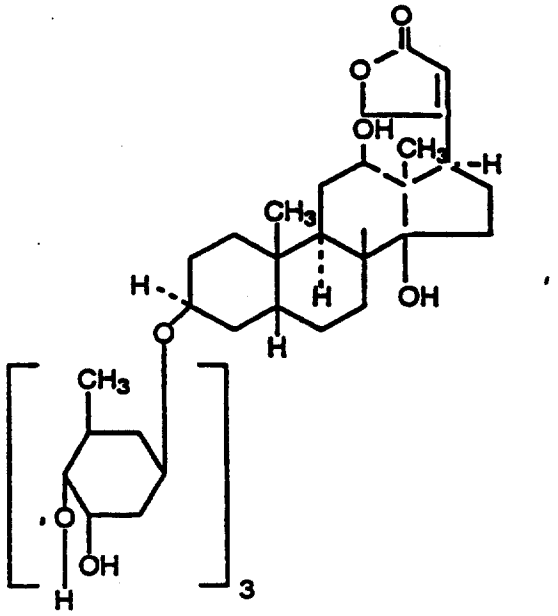
15. DATA SUMMARY OF digoxin

15.a Data Summary Charts:

Structure



Solubility

Table 1. Data Used in the Determination of Solubility of Digoxin in Distilled Water at 25 °C.

	Concentration (mg/mL)			
Sample	Hour 24–1	Hour 24–2	Hour 48–1	Hour 48–2
1–1	.0272	.0283	.0271	.0297
1–2	.0274	.0277	.0269	.0297
2–1	.0308	.0287	.0315	.0297
2–2	.0312	.0289	.0316	.0298
3–1	.0289	.0293	.0295	.0306
3–2	.0286	.0290	.0294	.0309

Table 2. Means, Standard Deviations and Percent Relative Standard Deviations for the Solubility of Digoxin (n=6)

	Solubility			
Sample	Mean (mg/mL)	Mean (M)	S. D.	% RSD
Water (24 hours)	.0288	3.69×10^{-5}	.0012	4.17
Water (48 hours)	.0297	3.80×10^{-5}	.0015	4.97
Water (overall)	.0293	3.75×10^{-5}	.0014	4.75

The water solubility of digoxin in distilled water is 3.75×10^{-6} M which is greater than 10^{-5} M. The aqueous solubility was not determined at pH 5.0, 7.0, and 9.0 because digoxin is not ionizable. Digoxin can be expected to affect the aquatic compartment based upon the water solubility value. Detailed information on this test is provided in Attachment II – Tier 0 – Physical and Chemical Data Report.

n–Octanol/Water Partition Coefficient

Table 3. Data from the Aqueous Phase

	Aqueous Concentration (mg/mL)	
Sample	Hour 24	Hour 48
1.88 mg		
Injection 1	.00330	.00303
Injection 2	.00315	.00290
6.07 mg		
Injection 1	.00997	.0102
Injection 2	.00997	.0102
9.25 mg		
Injection 1	.0153	.0155
Injection 2	.0152	.0155

Table 4. Organic Phase Data

	Organic Concentration (mg/mL)	
Sample	Hour 24	Hour 48
1.88 mg		
Injection 1	.0830	.0843
Injection 2	.0832	.0838
6.07 mg		
Injection 1	.261	.267
Injection 2	.263	.263
9.25 mg		
Injection 1	.399	.401
Injection 2	.391	.404

Table 5. Summary of Partition Coefficient Data for Digoxin

Sample	Initial Conc (M)	Aqueous Conc (M)	Organic Conc (M)	log K_{ow}	Average log K_{ow}
1.88 mg	1.20×10^{-4}	3.96×10^{-6}	1.07×10^{-4}	1.43	
6.07 mg	3.89×10^{-4}	1.29×10^{-5}	3.37×10^{-4}	1.42	1.42
9.25 mg	5.92×10^{-4}	1.97×10^{-5}	5.11×10^{-4}	1.41	

The determination of 1.42 for 1–octanol/water for the log K_{ow} is less than 2. Digoxin is not expected to affect the terrestrial compartment. Detailed information on this test is provided in Attachment II – Tier 0 – Physical and Chemical Data for digoxin.

Vapor Pressure Estimate

The vapor pressure estimate is 8.08×10^{-20} torr. Since the vapor pressure of digoxin is less tan 10^{-7} torr, it will not affect the atmospheric compartment. Detailed information on this estimate is provided in Attachment II – Tier 0 – Physical and Chemical Data for digoxin.

Dissociation Constant

Digoxin has no ionizable functional groups; therefore, no proton dissociation occurs and no dissociation constant can be determined.

UV Spectra

Table 6. UV Spectra Data in Distilled Water for digoxin.

Solvent	Wavelength (nm)	Character	A (1%, 1cm)	Molar Absorptivity $\varepsilon \times 10^{-4}$
Water				
Sample 1	220	maximum	179	1.40
Sample 2	220	maximum	187	1.46
0.1 *N* HCl Sample 1	220	maximum	172	1.34
0.1 *N* NaOH Sample 1	223.5	maximum	153	1.19

Table 7. Results of UV/Vis Spectra Determination of Digoxin in Various Solvents (ND = not determined because n = 1)

	Wavelength (nm)	Character	Average A (1%, 1 cm)	Range	Molar Absorptivity $\varepsilon \times 10^{-4}$	Range ($\times 10^{-2}$)
Average value in water						
	220	maximum	183	4	1.43	3
Average value in 0.1 *N* HCl						
	220	maximum	172	ND	1.34	ND
Average value in 0.1 *N* NaOH						
	223.5	maximum	153	ND	1.19	ND

The spectrum of digoxin shows slight shifts of the maximum in water and acidic and alkaline solutions. Detailed information on this test is provided in Attachment II – Tier 0 – Physical and Chemical Data for digoxin.

Hydrolysis Rate

At 37 °C, digoxin does not hydrolyze at pH 7 after 48 hours as reported in the literature (17). Detailed information on this test is provided in Attachment IV – References.

Photodegradation

Digoxin was one of three cardiac glycosides selected for photostability studies (18). The irradiation was carried out using a rotating reactor (Gräntzel/Karlsruhe), with a low–pressure mercury–lamp (254 nm) or day–light source. One gram of digoxin was mixed with 0.2 g 20% Aerosil 200. The mixture was put in a quartz glass tube and fixed to a rotating reactor. Dry nitrogen or synthetic air was circulated continuously throughout the procedure. An irradiation chamber (Heraeus/Hanau) with a high–pressure mercury–lamp (Q 300) was used for on–plate and petri dish methods. The irradiation was carried out for 96 hours, taking samples intermittently from the mixture every 24 hours.

Table 8. Yield of Photoproducts in Percents (%)

Compound	Irradiation Method	24 h	48 h	72 h	96 h
Digoxin	A	6.7	15.6	26.1	27.8
	B	3.2	12.7	25.7	26.9
	C	3.8	5.6	9.7	11.1
	D	0.0	0.9	1.2	1.6
	E	36.8			

A: Low-pressure mercury lamp (synthetic air); B: Low-pressure mercury lamp (nitrogen); C: High-pressure mercury lamp; D: Daylight source; E: On plate method.

Digoxin showed two main products on irradiation. Based on IR-, ^{1}H NMR- and ^{13}C NMR spectral data, the first product would appear to have a water group cleaved from the sugar moiety. The second product would appear to have the α, β-unsaturated lactone ring of the cardenolide cleaved. Detailed information on this test is provided in Attachment IV – References.

Daphnia magna Toxicity

A 24 hour EC_{50} was determined by Lilius, et al, for digoxin (19). This EC_{50} was reported as 0.031 ± 0.001 mM. See Attachment IV – References, for detailed information about this test.

15.b Attachments

ATTACHMENT I – Material Safety Data Sheet for Digoxin
ATTACHMENT II – Tier 0 – Physical and Chemical Data for Digoxin
ATTACHMENT III – Curricula Vitae of Authors
ATTACHMENT IV – References

15.c CONFIDENTIAL Appendices

CONFIDENTIAL: APPENDIX A – Identification of Chemical Substances
CONFIDENTIAL: APPENDIX B – Introduction of Substances Into The Environment

ATTACHMENT I

ATTACHMENT I:
MATERIAL SAFETY DATA SHEET
DIGOXIN

Burroughs Wellcome Co.	Intersection U.S. 13 & S.R. 1590 Post Office Box 1887 Greenville, North Carolina 27835-1887	Wellcome Cables & Telegrams Tabloid Greenville, N.C. TWX 5109291618 Tel. 919 758-3436

MATERIAL SAFETY DATA SHEET

GXSD/86/0015-1

SECTION I: IDENTIFICATION

Intermediate Names: DIGC; DIGR; DIGH; DIGS; DIGM; DIGQ; DIGG; DIGK; DIGU; DIGV

Product: Lanoxin

Chemical Name: 3β,5β,12β-3-[(0-2,6-Dideoxy-β-D-ribo-hexopyranosyl-(1→4)-0-2,6-dideoxy-β-D-ribo-hexopyranosyl)-(1→4)-2,6-dideoxy-β-D-ribo-hexopyranosyl]oxy]-12,14-dihydroxycard-20(22)-enolide

Formula: $C_{41}H_{64}O_{14}$ CAS #: 20830-75-5

Generic Name: Digoxin RTECS #: 1H6125000

For Information on Health Hazards Call: Ext 77014

Information Effective: 07/21/93

SECTION II: HAZARDOUS INGREDIENTS OF MIXTURES

Principal Hazardous Component(s): Digoxin

Toxicity: Oral LD_{50} (Guinea Pig) = 3.5 mg/kg[1] (Highly toxic per OSHA Hazard Communication Standard)
Oral LD_{50} (Cat) = 0.2 mg/kg[1]

SECTION III: PHYSICAL DATA

Boiling Point: NA Vapor Pressure: NA

Vapor Density: NA Solubility in Water: Insoluble

Specific Gravity (H_2O=1): NA Percent Volatile by Volume (%): NA

Evaporation Rate: NA Melting Point: 265° C

Appearance and Odor: Colorless, odorless crystals or a white or almost white crystalline powder with a bitter taste.

SECTION IV: FIRE AND EXPLOSION DATA

Flash Point (Method Used): ND Flammable Limits: LEL: ND UEL: ND

Special Fire Fighting Procedures: Self-contained breathing apparatus

Unusual Fire and Explosion Hazards: Combustible. Heating will give rise to toxic and irritant fumes

Extinguishing Media: Water spray, foam or dry agent

SECTION V: HEALTH HAZARD DATA

Threshold Limit Value: None established

Effects of Overexposure: Very toxic by inhalation and if swallowed in excess of the therapeutic dose. Digoxin is readily absorbed from the gastrointestinal tract and is rapidly distributed in the tissues. Eye contamination by dust can cause temporary visual disturbances. Toxic effects include nausea, vomiting, and cardiac irregularities. Overdoses cause vomiting, diarrhea, abdominal pain, yellow vision, low blood pressure, and irregular pulse; massive overdose will cause heart failure.

Routes of Entry: Can be ingested or inhaled.

Emergency and First Aid Procedures:

If in Eyes: Rinse immediately with plenty of water. Contact Health Center.
If Inhaled: Remove to fresh air. Rest and keep warm. Contact Health Center.
If Ingested: Wash out mouth. DIGIBIND^{R} may be administered to reduce toxic effects. Contact Health Center.
In Contact With Skin: Wash off with plenty of water. Contact Health Center if contact is prolonged.

SECTION VI: REACTIVITY DATA

Stability: Stable

Incompatibility (Materials to Avoid): NA

Conditions to Avoid: NA

Hazardous Decomposition Products: NA

Hazardous Polymerization: NA

SECTION VII: SPILL OR LEAK PROCEDURES

Steps to be Taken if Material is Spilled or Leaked: Wear suitable protective clothing. Sweep up, minimizing dust evolution and transfer to closed container for disposal. Clean area with detergent. Wash waters and rinses should be discharged to an approved sewerage system.

Waste Disposal Method: Consult Waste Disposal Procedures Manual or contact Environmental Services.

SECTION VIII: SPECIAL PROTECTION INFORMATION

Respiratory Protection: Toxic dust mask eg., 3M 8710.

Ventilation: Local exhaust or exhaust hood

Eye Protection: Goggles

Protective Gloves: Rubber

Other Protective Equipment: Air supplied respirator if dust is generated.

SECTION IX: SPECIAL PRECAUTIONS

Precautions to be Taken in Handling and Storage: Keep in original container tightly closed and dry. Protect from light.

Other Precautions: Wash hands after using.

SECTION X REFERENCES

[1]Substance Safety Information Sheet. GSS No: 0/1202/82

This information is supplied by Occupational Health and Safety Services to promote the safe use of chemicals through knowledge of the potential hazards. If you find any errors in the information in this Material Safety Data Sheet, contact Toby Holliday at extension 77014.

NA = Not Applicable
ND = Not Determined

ATTACHMENT II

ATTACHMENT II:
TIER 0 – PHYSICAL AND CHEMICAL DATA
DIGOXIN

BURROUGHS WELLCOME CO.
Greenville, NC, USA

Wellcome

GFZZ/95/0041

Digoxin: Environmental Assessment – Tier 0 Testing

M F S Whisler
Pharmaceutical Development Laboratories, Greenville, NC, USA

8 August 1995

Mary Fran Sewell Whisler
M F S Whisler

8/7/95
Date

CONTENTS

LIST OF TABLES

LIST OF FIGURES

DIGOXIN: ENVIRONMENTAL ASSESSMENT – TIER 0 TESTING

1. INTRODUCTION

The environmental assessment discussed in this report includes the required reporting information and the physical and chemical characteristics. The required data is stated in the *FDA Environmental Assessment Technical Assistance Handbook*, NTIS PB87-175345 and are listed in the Summary. Following that, the physical and chemical data are included; water solubility, the 1-octanol/water partition coefficient, vapor pressure, the ultraviolet-visible (UV/VIS) absorption spectra, and hydrolysis rate. They are presented in a summary format and then in detail. Unless stated in the individual sections of the report, the summary data that is listed applies to all of the individual tests.

2. SUMMARY

REQUIRED INFORMATION

Test chemical:	Digoxin
IUPAC name:	(3β,5β,12β)–3–[(*O*–2,6–Dideoxy–β–*D*–ribo–hexopyranosyl–(1→4)–*O*–2,6–dideoxy–β–*D*–ribo–hexopyranosyl–(1→4)–2,6–dideoxy–β–*D*–ribo–hexopyranosyl)oxy]–12,14–dihydroxycard–20(22)–enolide
CAS number:	[20830–75–5]
Trade name:	LANOXIN®
Empirical formula:	$C_{41}H_{64}O_{14}$
Manufacturer:	Burroughs Wellcome Co.
Lot number and purity of test substance:	DG1EM01 (98.2% and 98.9%) and DG1FM01 (98.1% and 97.7%)
Laboratory conducting test:	Burroughs Wellcome Co. Pharmaceutical Development Laboratories Physical Pharmacy P.O. Box 1887 Greenville, NC 27835–1887

3. PHYSICAL AND CHEMICAL DATA

3.1 Water Solubility (Environmental Assessment Technical Document 3.01)

Water 3.75×10^{-5} M

The water solubility of digoxin is relatively low, but greater than the criteria concentration of 1×10^{-5} M. This suggests that digoxin may affect the aquatic compartment.

3.2 1-Octanol/Water Partition Coefficient (Environmental Assessment Technical Document 3.02)

$K_{OW} = 1.42$

The log P value is less than 2: therefore, digoxin is not expected to significantly bioconcentrate or sorb onto organic particles. The terrestrial compartment should not be directly affected.

3.3 Vapor Pressure Estimation (Environmental Assessment Technical Document 3.03)

8.08×10^{-20} torr

The vapor pressure estimate is less than 10^{-7} torr. Digoxin is not expected to affect the atmospheric compartment.

3.4 Dissociation Constant (Environmental Assessment Technical Document 3.04)

Digoxin has no ionizable functional groups; therefore, no proton dissociation occurs and no dissociation constant can be determined.

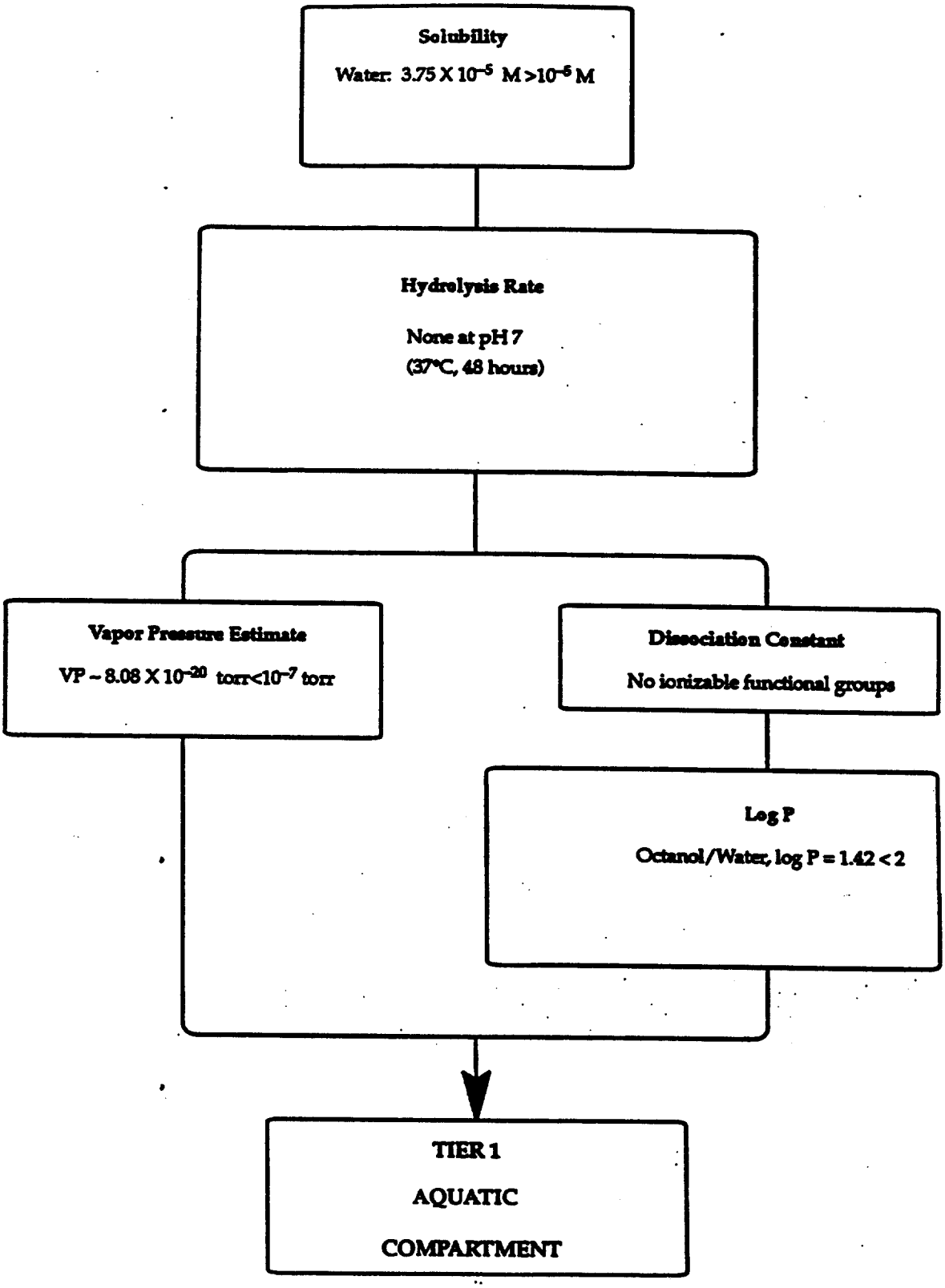
3.5 UV Absorption Spectra (Environmental Assessment Technical Document 3.05)

Dependent on the solvent or solution studied, digoxin is a UV absorber between 219 and 224 nm.

3.6 Hydrolysis (Environmental Assessment Technical Document 3.09)

At 37 °C, digoxin does not hydrolyze at pH 7 after 48 hours as reported in the literature[1]; therefore, no additional testing was done.

TIER 0 Results for Digoxin



4. WATER SOLUBILITY

Responsible Scientist:	Richard Winnike
Dates of Testing:	28 August 1991 to 19 March 1992
References:	Wade, D. Burroughs Wellcome Notebook: 91/5432 and 91/5701
	Winnike, R. Burroughs Wellcome Notebook: 91/5127

4.1 Research Objective

The purpose of this study is to determine the solubility of digoxin in aqueous media. The solubility of a solid or a liquid chemical can be defined as the maximum amount of the chemical (the solute) in solution at equilibrium with excess solid chemical in a solvent at specified ambient conditions (temperature, atmospheric pressure, and pH).

4.2 Background

If the solubility is less than 10 parts per billion (ppb) or 0.00001 mg/mL no quantitation is necessary and solubility can be reported as less than 10 ppb. If the solubility is greater than 10 mg/mL, the density of the test solution must be determined at the temperature of interest.

Required pretest information needed includes the method for analysis of the test chemical in water, the dissociation in water, and the purity of the test chemical.

4.3 Procedure

The saturated solubility should be determined from above and below saturation. Only one set of samples was prepared for this solubility determination by approaching saturation from below saturation. This was accomplished by placing distilled water in an appropriate container and adding sufficient test chemical so that the solution was saturated. The containers were sealed and allowed to equilibrate at 25 °C with shaking. The samples were checked periodically to insure that the solutions remained saturated. If not, additional test chemical was added to maintain saturation. Once the samples remained saturated, an aliquot was removed after shaking for 24 hours and filtered and analyzed. Additional samples were analyzed at 48 hours when equilibrium was achieved.

The aqueous solubility was not determined at pH 5, 7, or 9, because digoxin is not ionizable.

4.4 Instruments

Blue M Magni Whirl Constant Temperature Bath

HPLC including:

SpectroPhysics Model SP8770 pump with a Valco valve and a 10 µL injection loop
Kratos Spectroflow Model 757 detector
Micromeritics 725 Autoinjector
Linear recorder
Spherisorb ODS–1, 5 µm, 150 mm x 4.6 mm (ID) column
Brownlee Guard Column RP18, 7 µm, 3.2 mm ID
Mobile phase: 26% (v/v) acetonitrile in distilled water
Hewlett Packard H/P 3357 system for data collection and integration

4.5 Data

Table 1. Data used in the determination of solubility of digoxin

	Concentration (mg/mL)			
Sample	Hour 24–1	Hour 24–2	Hour 48–1	Hour 48–2
1–1	.0272	.0283	.0271	.0297
1–2	.0274	.0277	.0269	.0297
2–1	.0308	.0287	.0315	.0297
2–2	.0312	.0289	.0316	.0298
3–1	.0289	.0293	.0295	.0306
3–2	.0286	.0290	.0294	.0309

Table 2. The means, standard deviations (n–1), and percent relative standard deviations for the solubility of digoxin

	Solubility			
Sample	Mean (mg/mL)	Mean (M)	S. D.	% RSD
Water (24 hours)	.0288	3.69×10^{-5}	.0012	4.17
Water (48 hours)	.0297	3.80×10^{-5}	.0015	4.97
Water (overall)	.0293	3.75×10^{-5}	.0014	4.75

4.6 Discussion

Digoxin has a very low solubility in water. Duplicate injections of triplicate samples were analyzed to determine the value for this parameter. The value reported is the mean of the twenty–four individual determinations.

4.7 Conclusion

Water 3.75×10^{-5} M or
2.93×10^{-2} mg/mL

Digoxin has very limited aqueous solubility; therefore, it may affect the aquatic compartment.

5. 1–OCTANOL/WATER PARTITION COEFFICIENT

Responsible Scientist:	Richard Winnike
Dates of Testing:	8 August 1991 to 10 September 1991
References:	Wade, D. Burroughs Wellcome Notebook: 91/5432

5.1 Research Objective

The purpose of this study is to determine the partition coefficient of digoxin in 1–octanol and water. The 1–octanol/water partition coefficient is the ratio of the concentrations of a given chemical species in the two phases at equilibrium; the test chemical is in dilute solution in both phases. That is, the concentration of the chemical is normally well below 0.01 M in either phase. This ratio is a constant at any given temperature, assuming no significant associations or dissociations of the solute in each phase. Solute dissociations and solute–solute associations result in the presence of more than one molécular species, such as ions and dimers. 1–Octanol is considered a good medium for simulating natural fatty substances.

5.2 Background

Pretest information that is needed to conduct this test is the water solubility of the test chemical, the dissociation constant, evaluation of potential for rapid hydrolysis of the test chemical, and the method of analysis of quantification. If the test chemical is ionizable, the partition coefficient should also be determined using 1–octanol and water at pH 5, 7, and 9.

5.3 Procedure

To determine the 1–octanol/water partition coefficient, highly purified water and 1–octanol were presaturated by agitation for at least one hour. Triplicate samples at three concentrations of the test chemical were tested. The initial concentrations in the 1–octanol phase covered a five fold range. A known quantity of the test chemical was dissolved in the 1–octanol because it dissolves the chemical more readily; then the distilled water was added. The samples were shaken at the controlled temperature of 25 ±1 °C in an appropriate container. After 24 hours, the samples were centrifuged to separate the two phases. A portion was pipetted from the aqueous and organic phase of each sample and assayed by HPLC. The samples were shaken for 24 more hours and the same procedure repeated to determine the test chemical concentration. Since digoxin has no ionizable functional groups, the partition coefficient would not be expected to change as a function of aqueous phase pH.

5.4 Instruments

Blue M Magni Whirl Constant Temperature Bath

HPLC including

SpectroPhysics Model SP8770 pump with a Valco valve and a 10 µL injection loop
Kratos Spectroflow Model 757 detector
Micromeritics 725 Autoinjector
Linear Recorder
Spherisorb ODS–1, 5 µm, 150 mm x 4.6 mm (ID) column
Brownlee Guard Column RP18, 7 µm, 3.2 mm ID
Mobile phase: 26% (v/v) acetonitrile in distilled water
Hewlett Packard H/P 3357 system for data collection and integration

5.5 Data

Table 3. Data from the aqueous phase

	Aqueous Concentration (mg/mL)	
Sample	Hour 24	Hour 48
1.88 mg		
Inj 1	.00330	.00303
Inj 2	.00315	.00290
6.07 mg		
Inj 1	.00997	.0102
Inj 2	.00997	.0102
9.25 mg		
Inj 1	.0153	.0155
Inj 2	.0152	.0155

Table 4. Data from the organic phase

	Organic Concentration (mg/mL)	
Sample	Hour 24	Hour 48
1.88 mg		
Inj 1	.0830	.0843
Inj 2	.0832	.0838
6.07 mg		
Inj 1	.261	.267
Inj 2	.263	.262
9.25 mg		
Inj 1	.399	.401
Inj 2	.391	.404

5.6 Calculations

Calculate the apparent partition coefficient (P) as

$$P = C_1/C_2$$

where: P is the apparent partition coefficient
C_1 is the molar concentration in 1–octanol
C_2 is the molar concentration in water

NOTE: P is also referred to as K_{OW}

For example, to calculate the apparent partition coefficient (P):

(1.88 mg –Inj 1) $P = (1.06 \times 10^{-4} M)/(4.18 \times 10^{-6} M)$
$P = 25.4$

For example, to calculate the log K_{ow} take the log of P

(1.88 mg Inj 1) $\log K_{ow} = \log 25.4$
$\log K_{ow} = 1.41$

5.7 Discussion

The partition coefficient was determined using three different concentrations of digoxin. The agreement between the numbers confirm that the value is a constant for this unionizable molecule.

5.8 Conclusion

Table 5. Results of partition coefficient of digoxin

Sample	Initial Conc. (M)	Aqueous Conc. (M)	Organic Conc. (M)	log K_{ow}	Average log K_{ow}
1.88 mg	1.20×10^{-4}	3.96×10^{-6}	1.07×10^{-4}	1.43	
6.07 mg	3.89×10^{-4}	1.29×10^{-5}	3.37×10^{-4}	1.42	1.42
9.25 mg	5.92×10^{-4}	1.97×10^{-5}	5.11×10^{-4}	1.41	

The determination of 1.42 for 1–octanol/distilled water for the log K_{ow} is less than the K_{ow} of 2.

Digoxin is not expected to affect the terrestrial compartment.

6. VAPOR PRESSURE ESTIMATION

Responsible Scientist:	Robin Currie
Dates of Estimation:	26 June 1995
References:	Currie, R. Burroughs Wellcome Notebook: 95/5054
	Lyman WJ, Reehl WF, Rosenblat DH. Handbook of chemical property estimation methods. Washington, DC: American Chemical Society, 1990.

6.1 Research Objective

The purpose of this study is to estimate the vapor pressure of digoxin. The vapor pressure of a test chemical governs the tendency to be transported in air; thus, it is a predictor of the distribution of chemicals in different environmental compartments. Additionally, vapor pressure values can be used to prevent or account for the loss of volatile materials during other tests.

6.2 Background

The vapor pressure should be used in conjunction with values of other fate parameters to decide if other tests are needed for a more complete description of the test chemical's environmental fate.

6.3 Procedure

Vapor pressure can be estimated by a variety of methods. The calculations were performed using the Clausieus–Clapeyron equation and the modified Watson correlation for solids. This method used the chemical structure and the normal boiling point in the following equation. This value was calculated and reported.

6.4 Calculations

Table 6. Boiling point(T_b) by Meissner's method: $T_b = (637 \times [R_D]^{1.47} + B)/[P]$

Atom	Number	[Rn]	[P]	Calculated [Rn]	Calculated [P]
Carbon	41	2.418	9.2	99.138	377.2
Hydrogen					
(on Nitrogen)		1.1	12.5	0	0
(on Carbon)	56	1.1	15.4	61.6	862.4
(on oxygen)	8	1.1	10	8.8	80
Oxygen					
(on hydroxyl)	6	1.525	20	9.15	120
(on Carbonyl)		2.211	39	0	0
(in ether)	6	1.643	20	9.858	120
(in ester)	1	3.736	54.8	3.736	54.8
Chlorine		5.967	55	0	0
Nitrogen					
(Tertiary/in ring)		2.84	17.5	0	0
(Secondary)		2.502	17.5	0	0
(Primary)		2.322	17.5	0	0
6 membered ring	6	0	0.8	0	4.8
5 membered ring	2	0	3	0	6
Double bonds	1	1.733	19	1.733	19
Singlet linkage		0	-9.5	0	0
Strain					
RCOOR	1	0	-3	0	-3
RNH2		0	0	0	0
R2CHR		0	-3	0	0
R2NH		0	-3	0	0
Ring Carbonyl	1	0	3	0	3
R3N		0	-6	0	0
TOTAL				$[R_D]$ =194.015	[P] = 1644.2

Calculations For the Constant "B"

COMPOUND CLASS	Number	B	Calculated B
Acid(monocarboxylic)		28000	0
Alcohols		16500	0
Primary Amine		6500	0
Secondary Amine		2000	0
Tertiary Amine		-3000	0
Esters(monocarboxylic acid)		15000	0
Esters(dibasic acid)		30000	0
Ethers and mercaptans	1	4000	4000
TOTAL			4000

Therefore T_b is 896 K T_b = 623 °C

Vapor Pressure: Clausius–Clapeyron equation:

$$\ln P_{VP} = \left[\Delta H_{vb} (T_b - C_2)^2 / \Delta Z_b R T_b^2\right]\left[(1/(T_b - C_2)) - (1/(T - C_2))\right]$$

Where $\Delta H_{vb}/T_b = KF\ (8.75 + R \ln T_b)$

$C_2 = -18 + 0.19\ T_b$

$\Delta Z_b = 0.97$

$T_b = 896$ K

$R = 1.987$

$KF = 1.05$

$\ln P_{VP} = -50.5958804$

P_{VP} (atm) = 1.0629E–22

P_{VP} (torr) = 8.08E–20

6.5 Conclusion

Because the vapor pressure was estimated by calculation to be less than 10^{-7} torr, no further testing was needed. Digoxin will not affect the atmospheric compartment.

7. DISSOCIATION CONSTANT

Responsible Scientist:	Richard Winnike
References:	Winnike, R. and Kidd, C. Internal Document, Burroughs Wellcome Co., GFFD/92/0001, March 31, 1992.

7.1 Conclusion

Digoxin has no ionizable functional groups; therefore, no proton dissociation occurs and no dissociation constant can be determined.

8. ULTRAVIOLET ABSORPTION SPECTRA

Responsible Scientist:	Richard Winnike
Dates of Testing:	17 January 1992
References:	Winnike, R. Burroughs Wellcome Notebook: 91/5127

8.1 Research Objective

The purpose of this study is to measure the ultraviolet/visible (UV/VIS) absorption spectra of digoxin. Absorption spectra give some indication of the different wavelengths at which a test chemical can be subjected to direct photodegradation. Before a chemical can undergo a direct photochemical reaction, it has to be exposed to light and have the ability to absorb energy from wavelengths in this referenced spectrum. Photodegradation will depend on the total energy absorbed and is characterized by both the molar extinction coefficient (absorptivity) and the band width. However, the absence of measurable absorption does not preclude the possibility of photodegradation by other means. The ultraviolet–visible spectrum of a chemical is characteristic of the molecular structure. Each chemical has a unique spectrum that can be used for identification of the pure chemical. For test chemicals that reversibly ionize or protonate, the absorption spectrum should be obtained in acid solutions, below pH 5, and in basic solutions, above pH 9. These conditions are representative of environmental conditions.

8.2 Background

Pretest information needed for this test includes the water solubility, the dissociation constant in water and the vapor pressure.

8.3 Procedure

The absorption spectrum for digoxin in the ultraviolet range should were obtained at ambient conditions. The test chemical was dissolved in water, 0.1 *N* HCl, and 0.1 *N* NaOH. The spectrum was scanned from 200 and 400 nm. The molar absorptivity and absorbance (1%, 1 cm) were calculated from the absorbance and concentration of the dilute solutions. The results are reported in Table 8.

8.4 Instrument

Perkin Elmer Lambda 6 UV/VIS spectrophotometer, 1 cm cell path, ambient room temperature

8.5 Data

Table 7. UV data of digoxin in various solvents

Solvent	Wavelength (nm)	Character	A (1%, 1 cm)a	Molar Absorptivity(ε) ($\times 10^{-4}$)
Water				
Sample 1	220	maximum	179	1.40
Sample 2	220	maximum	187	1.46
0.1 *N* HCl				
Sample 1	220	maximum	172	1.34
0.1 *N* NaOH				
Sample 1	223.5	maximum	153	1.19

[a] The specific absorbance, A (1%, 1 cm) as defined in the British Pharmacopoeia, Volume II, is the "absorbance of 1–cm layer of a 1% w/v solution of the absorbing solute, its value at a particular wavelength and in a given solvent being a property of the solute".
Thus, A (1%, 1 cm) = A/cd

where A (1%,1cm) is the specific absorbance, A is the absorbance defined by the expression, $A = \log_{10}(1/T)$,
c is the concentration of the absorbing solute expressed as a percentage w/v,
d is the thickness of the absorbing layer in cm, and
T is the fraction of the incident radation transmitted by the sample solution.

The spectrum of digoxin shows slight shifts in the maxima in various solutions. See Figures 1, 2, and 3 for the spectra.

Table 8. Results of UV/VIS spectra determinations of digoxin in various solvents (ND = not determined because n = 1)

	Wavelength (nm)	Character	Average A (1%, 1 cm)	Range	Average Molar Absorptivity(ε) ($\times 10^{-4}$)	Range ($\times 10^{-2}$)
Average value in water						
	220	maximum	183	4	1.43	3
Average value in 0.1 *N* HCl						
	220	maximum	172	ND	1.34	ND
Average value in 0.1 *N* NaOH						
	223.5	maximum	153	ND	1.19	ND

8.6 Conclusion

The spectrum of digoxin shows slight shifts of the maximum in water and acidic and alkaline solutions.

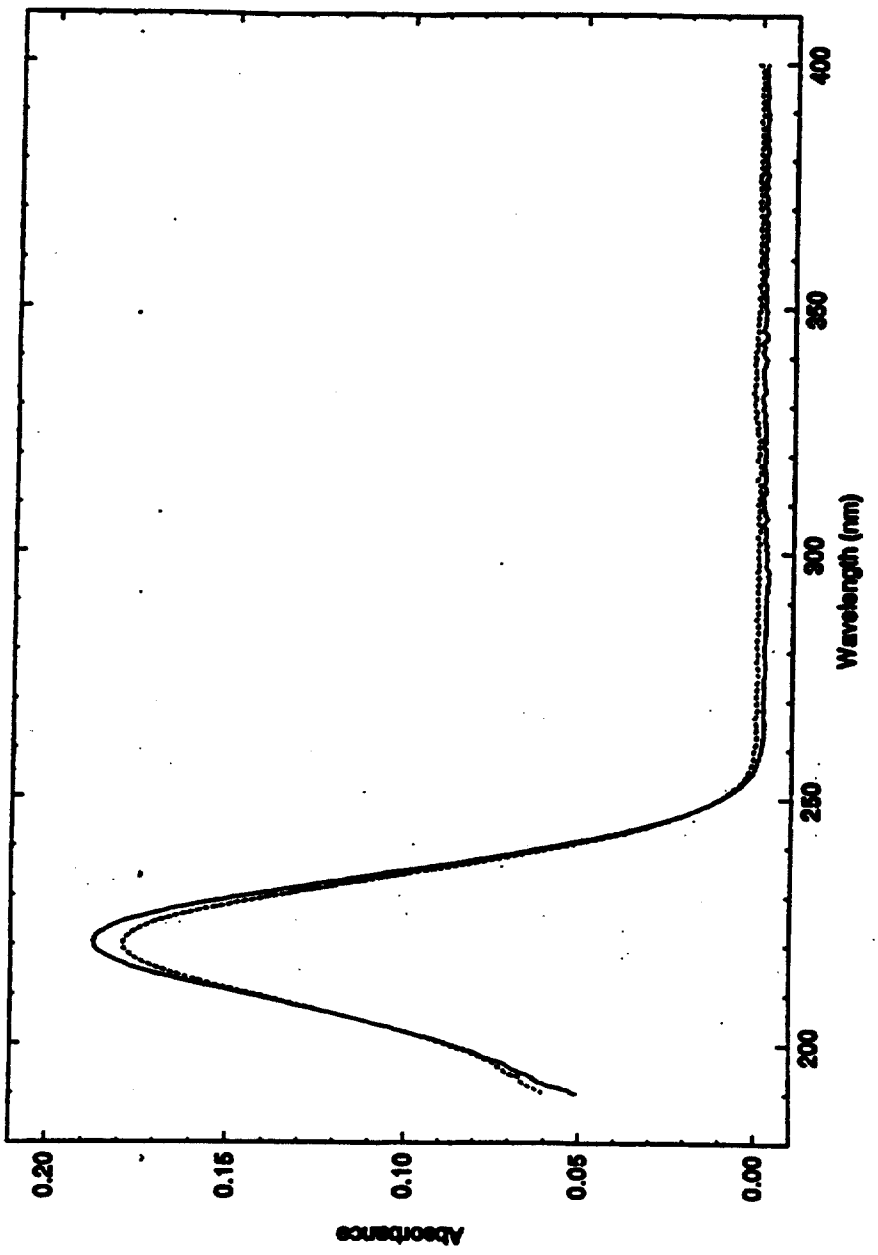


Figure 1. UV scan of digoxin in distilled water, Samples 1 and 2

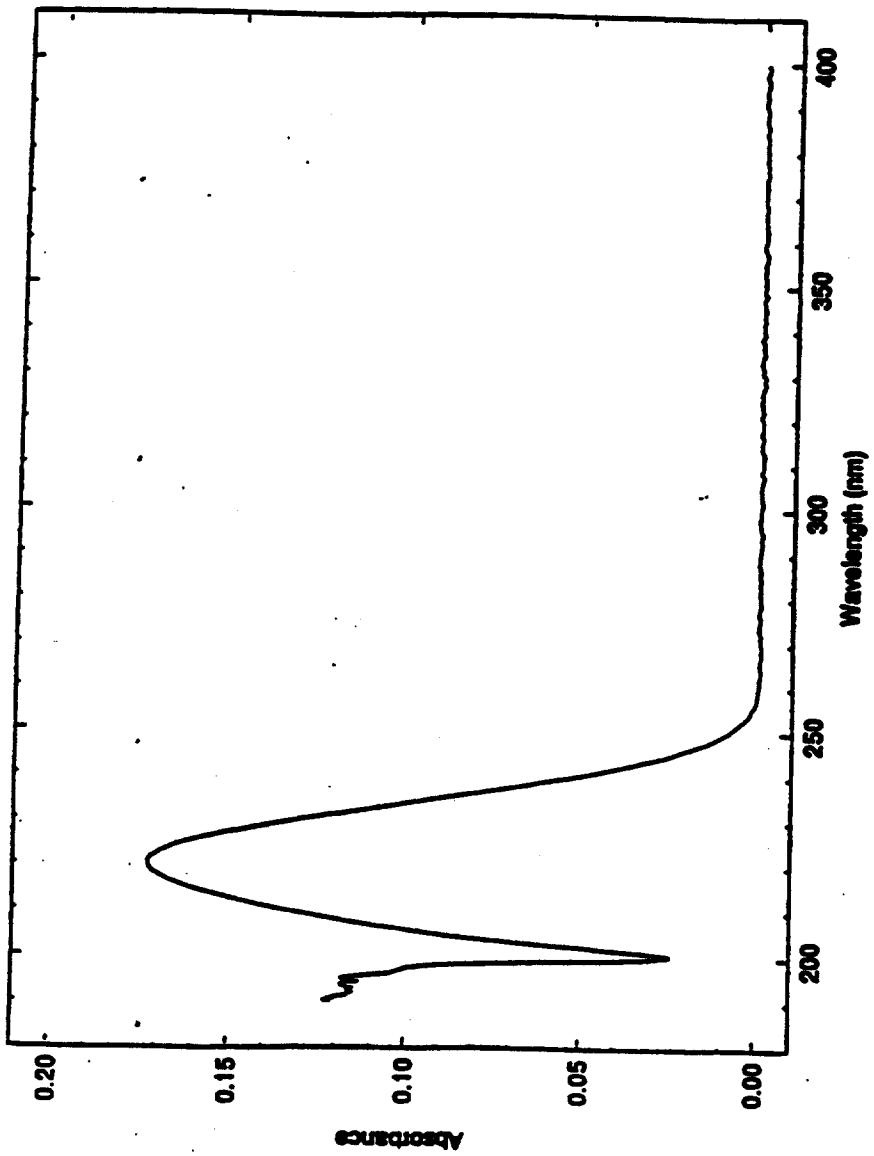


Figure 2. UV scan of digoxin in 0.1 *N* HCl

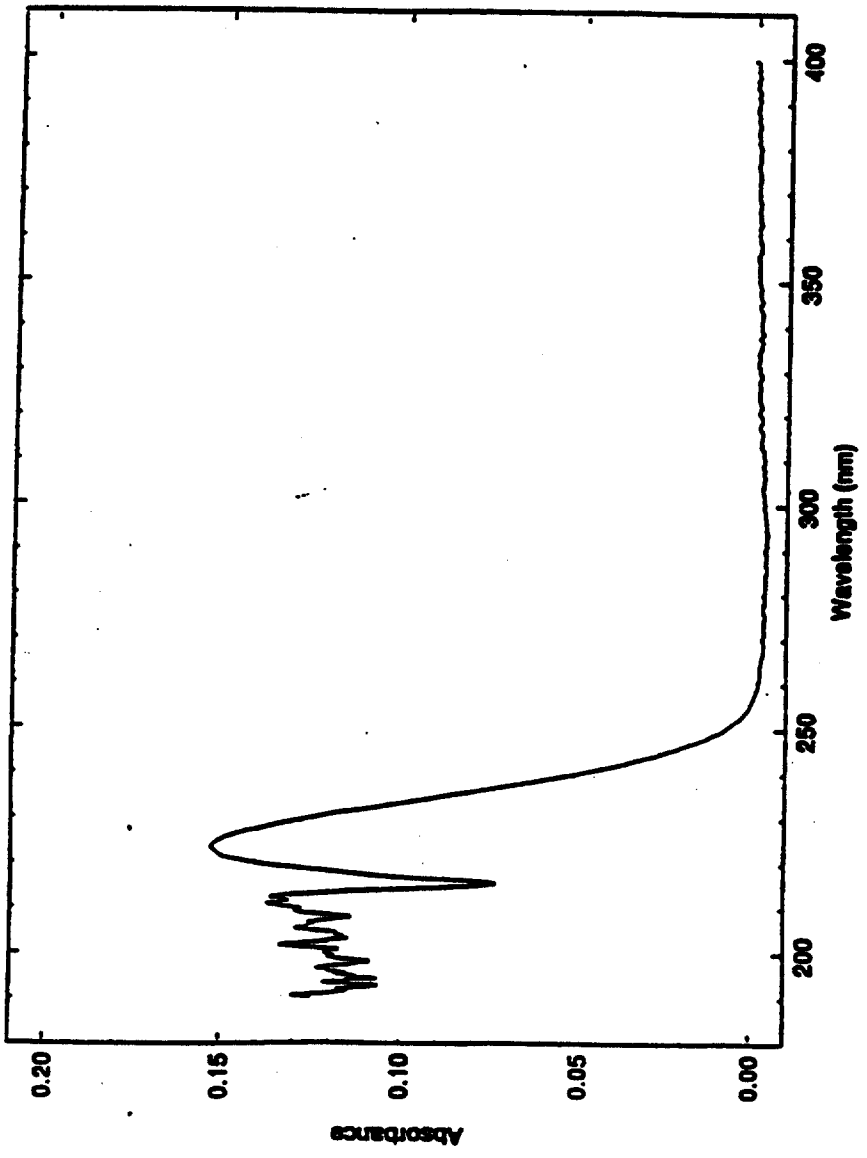


Figure 3. UV scan of digoxin in 0.1 *N* NaOH

9. HYDROLYSIS

9.1 Conclusion

At 37 °C, digoxin does not hydrolyze at pH 7 after 48 hours as reported in the literature[1]; therefore no additional testing was done.

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ATTACHMENT III

ATTACHMENT III:
CURRICULA VITAE OF AUTHORS

CURRICULUM VITAE

BRENDA BUNCH FRANKLIN

Technical Operations Specialist
Chemical Development Laboratories

EDUCATION

School	Specialty	Degree	Year
University of Missouri–Columbia	Chemistry	B.S.	1981

PROFESSIONAL ORGANIZATIONS

American Chemical Society
Society of Environmental Toxicology and Chemistry
Alpha Chi Sigma

EXPERIENCE

Company/Institution	Position	Year(s)
Midwest Research Institute, Kansas City, MO.	Analytical Chemist,	1982–1984
ABC Laboratories, Columbia, MO	Supervisor, Analytical Support	1984–1988
ABC Laboratories, Columbia, MO	Group Leader, Aquatic Toxicology Group	1988–1990
ABC Laboratories, Columbia, MO	Manager, Aquatic Toxicology Division,	1990–1991
ABC Laboratories, Columbia, MO	Senior Manager, Aquatic Toxicology Programs,	1991–1993
Burroughs Wellcome Co.	Technical Operations Specialist	1993–Present

PUBLICATIONS

Adams, W.J., W.J. Rehandette, J. D. Doi, M. G. Stepro, M. W. Tucker, R. A. Kimerle, B. B. Franklin, J. V. Nabholz. "Experimental Freshwater Microcosm Biodegradability Study of Butyl Benzyl Phthalate," *Aquatic Toxicology and Environmental Fate: Eleventh Volume, ASTM STP 1007*, B. W. Suter II and M.A. Lewis, Eds., American Society for Testing and Materials, Philadelphia, pp. 19–40.

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Franklin, B. B., B. Q. Litherland. "Environmental Partnership Programs Between Industry and Education," Interface '93, Tan–Tar–A Resort, Osage Beach, MO. Paper 225.

CURRICULUM VITAE

SARAH R. ALSTON

Environmental Engineer
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EDUCATION

School	Specialty	Degree	Year
Louisiana State University	Civil Engineering,	M.E..	1982
Louisiana State University	Microbiology	B.S.	1979

SOCIETIES AND SCHOLARSHIPS

Four-year scholarship to LSU for "Best in Rally," State Speech Rally, 1974

American Chemical Society, Chairman, Safety Committee, North Carolina Section (1987)

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EXPERIENCE

Company/Institution	Position	Year(s)
EPA Environmental Photographic Interpretation Center (EPIC), Warrenton, VA	Environmental Specialist	Summer 1980
Department of Civil Engineering, LSU	Research Assistant	January 1980 to August 1982
N.C. Solid & Hazardous Waste Management Branch	Environmental Engineer	March 1983 to February 1984,
Burroughs Wellcome Co., Research Triangle Park, NC.	Environmental Operations Supervisor	March 1984 to December 1990,
Burroughs Wellcome Co., Greenville, NC.	Environmental Project Engineer, Environmental Services Dept.	December 1990 to present,

PUBLICATIONS

"Water Related Problems in the Coastal Zone," with R. F. Malone, M. E. Tittlebaum, S. M. Crane, and N. S. Sanders (1981)

"Remote Sensing and Detection of Hazardous Waste Sites," with C. A. Harlow et. al., Remote Sensing and Image Processing Laboratory, LSU (1982)

Thesis Title: "Development of a Methodology for Evaluating Waste Disposal Sites" funded by the Office of Water Research & Technology (1982)

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CURRICULUM VITAE

MARY F. S. WHISLER

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EDUCATION

School	Specialty	Degree	Year
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The Ohio State University Columbus, Ohio	Pharmacy	B.S.	1976
University of Kentucky Lexington, Kentucky	Pharmaceutical Sciences	Ph.D.	1982

PROFESSIONAL ORGANIZATIONS

American Pharmaceutical Association

Academy of Pharmaceutical Research and Science

American Association of Pharmaceutical Scientists

Rho Chi Society

Controlled Release Society

North Carolina Pharmaceutical Discussion Group

EXPERIENCE

Company/Institution	Position	Year(s)
Anderson's Drug Store, Chillicothe, Ohio	Registered Pharmacist	1976 – 1978
University of Kentucky, Lexington, Kentucky	Graduate Teaching Assistant	1978 – 1980
University of Kentucky, Lexington, Kentucky	Graduate Research Assistant	1980 – 1982
Burroughs Wellcome Co., Greenville, NC	Development Scientist, Pharmaceutical Development Laboratories	1982 – present

PUBLICATIONS

Miles, Michael V., Balasubramanian, Ramadas, Pittman, A. Wayne, Grossman, Steven H., Pappa, Keith A., Smith, Mary Faye, Wargin, William A., Findlay, John W. A., Poust, Rolland L., Frosolono, Michael F., "Pharmacokinetics of Oral and Transdermal Triprolidine", J. Clin. Pharmacol 30, 572 (1990).

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Smith, M.F. and Digenis, G.A., "Synthesis of Esters of Haloperidol", The Academy of Pharmaceutical Sciences Meeting, Orlando, Florida, 1981.

CURRICULUM VITAE

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QUALIFICATIONS

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EXPERIENCE

1976–1981	Analyst for Northumbrian Water Authority.
1983–1986	Analyst for Welsh Water Authority.
1986–1988	Analytical and Consultant Chemist, Rooney Laboratories, Basingstoke.
1988–1990	Analyst, Environmental Management, Wellcome Foundation Ltd., Dartford.
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CURRICULA VITAE

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EDUCATION	**School**	**Specialty**	**Degree**	**Year**
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PROFESSIONAL ORGANIZATIONS	Order of Engineers of Quebec			

EXPERIENCE	**Company/Institution**	**Position**	**Year(s)**
	Burroughs Wellcome Inc. Kirkland, Quebec, Canada	Validation Specialist	1985–1987
	Burroughs Wellcome Inc. Kirkland, Quebec, Canada	Project Engineer	1987–present

ATTACHMENT IV:
REFERENCES

bonate bands are compared (Table II). The carbonate bands observed in the calcium carbonate antacid are very similar to those of the unperturbed carbonate ion, indicating a symmetrical arrangement of carbonate in the crystal structure.

A significant perturbation of the carbonate IR absorption bands was observed in carbonate-containing aluminum hydroxide gel, reflecting the coordination of carbonate to aluminum in the amorphous structure. The splitting of the ν_3 vibration suggests a unidentate interaction with aluminum. Carbonate is perturbed to a greater degree in dihydroxyaluminum sodium carbonate, suggesting a bidentate coordination for the carbonate ion. This is in good agreement with the crystal structure of dawsonite.

IR evidence suggests that both carbonate and bicarbonate are interlayer anions in hydrotalcite.

Based on IR and X-ray analysis, magaldrate has a hydrotalcite-like structure with sulfate as the major interlayer anion and carbonate present in the interlayer space. The evidence does not support the present identification of magaldrate as a magnesium aluminum hydroxide.

The relationship between the carbonate-containing compounds used as antacids and natural minerals is also apparent based on this study. Amorphous carbonate-containing aluminum hydroxide gel can be classified mineralogically as amorphous aluminum hydroxycarbonate. The crystalline carbonate-containing antacids are somewhat poorly organized forms of the minerals calcite, $CaCO_3$; dawsonite, $NaAl(OH)_2CO_3$; and hydrotalcite, $Mg_6Al_2CO_3(OH)_{16}\cdot 4H_2O$.

Based on these findings, it is suggested that the official definitions should recognize that: (*a*) carbonate is coordinated to aluminum in the aluminum hydroxide gel structure; (*b*) dihydroxyaluminum sodium carbonate is a synthetic form of the mineral dawsonite and the mineralogical formula $NaAl(OH)_2CO_3$ should be used rather than the present official formula, $CH_2AlNaO_5\cdot xH_2O$, since no molecular water is present and the official formula does not clearly indicate that the compound is a hydroxycarbonate; and (*c*) magaldrate has a hydrotalcite-like structure.

It is believed that an understanding of the structural relationship between carbonate-containing antacids will lead to improved methods for the production and control of the antacids and will provide a useful framework for the development of new antacids.

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This report is Journal Paper 6821, Purdue University Agricultural Experiment Station, West Lafayette, IN 47907.

Kinetics of Digoxin Stability in Aqueous Solution

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Received October 18, 1976, from the [a]*Department of Pharmaceutical Chemistry, McCollum Laboratories, University of Kansas, Lawrence, KS 66044*, and the [x]*INTERx Research Corporation, Lawrence, KS 66044.* Accepted for publication June 13, 1977.

Abstract □ Digoxin hydrolysis was studied as a function of pH. Conversion of digoxin to digoxigenin was followed by high-pressure liquid chromatography and shown to proceed by the initial loss of one, two, or three sugars. The hydrolysis rate was directly proportional to parent drug concentration and hydrogen-ion activity. The individual hydrolysis rate constants of digoxin, digoxigenin bisdigitoxoside, and digoxigenin monodigitoxoside were determined by a simplex fitting procedure. Data are presented suggesting that at least some variation in the bioavailability of orally administered digoxin arises from observed variations in gastric pH; these variations influence the extent to which hydrolysis occurs and, thus, modify the composition of digoxin species available for absorption.

Keyphrases □ Digoxin—hydrolysis, kinetic study, effect of pH □ Hydrolysis—digoxin, kinetic study, effect of pH □ Kinetics—digoxin hydrolysis, effect of pH □ Stability—digoxin in aqueous solution, kinetic study of hydrolysis, effect of pH □ Cardiotonic agents—digoxin, hydrolysis, kinetic study, effect of pH

Digoxin is a widely prescribed glycoside used in the maintenance therapy of cardiac patients. Significant variability has been observed in the efficiency with which digoxin is absorbed from the GI tract among patients given the drug orally. Moreover, significant variations in bioavailability were noted within individual patients (1, 2).

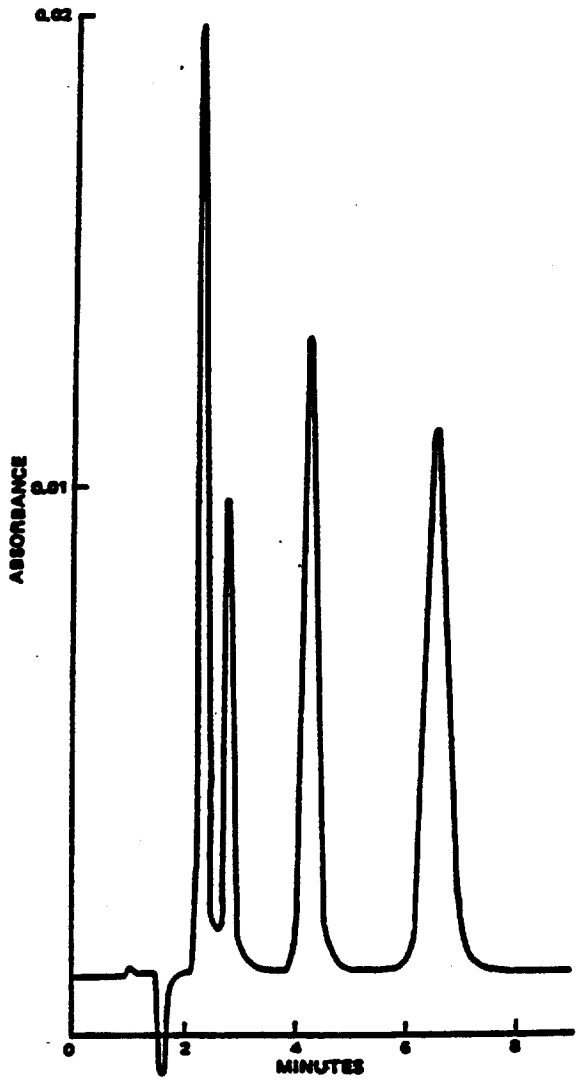


Figure 1—*Chromatogram of digoxigenin species separated by reversed-phase HPLC, obtained from an aqueous solution containing IV (t_R = 2.2 min; 12.2 μg/ml), III (t_R = 2.6 min; 10.2 μg/ml), II (t_R = 4.1 min; 30 μg/ml), and I (t_R = 6.4 min; 4.16 μg/ml).*

Potential sources for this variability have been postulated to involve dosage formulation and inconsistencies in administration (3–7). However, investigations of these parameters have failed to explain many observations associated with oral digoxin absorption.

The glycosidic nature of the digoxin molecule makes it susceptible to hydrolysis, especially in the acidic environment of the stomach. If hydrolysis is rapid enough to compete with absorption, fluctuations in bioavailability may reflect alterations in the amount and composition of the absorbed species. The hydrolysis products have been shown (8) to exhibit different pharmacological and toxicological activity than digoxin but are not distinguished from the parent drug using presently available clinical analytical methods.

Therefore, a study was initiated to determine if the variability in digoxin absorption could arise from variations in the extent to which hydrolysis occurs among patients and, thus, competes with drug absorption. An analytical method was developed to quantitate digoxin and its three potential hydrolysis products simultaneously. This method was used to study the hydrolysis kinetics of digoxin species under acid conditions, similar to those found during oral absorption.

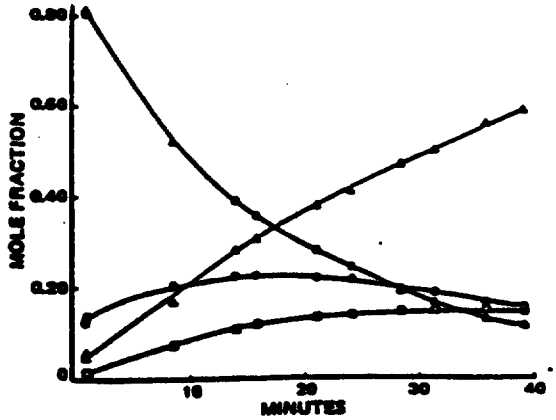


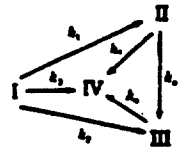
Figure 2—*Time–concentration profile of digoxigenin species [I (△), II (○), III (□), and IV (△)] present in an aqueous solution of I at pH 1 incubated at 37°.*

RESULTS AND DISCUSSION

Digoxin (digoxigenin tridigitoxoside) hydrolysis was studied at 37° at pH 1, 2, 4, and 7. At pH 7, no appreciable hydrolysis was observed over 48 hr. In all other media, four-component mixtures were generated, corresponding to the cleavage of one, two, or three digitoxose residues. Separation and simultaneous quantitation of each of the four components in the hydrolysis mixtures were effected by reversed-phase high-pressure liquid chromatography (HPLC) (Fig. 1). Twenty-five nanograms of digoxigenin species could be determined by monitoring the eluent spectrophotometrically at the λ_{max} (225 nm) for digoxin species.

Digoxin (I) hydrolysis is a complex combination of parallel reactions that can proceed initially by three alternative pathways to yield digoxigenin (IV), which is unreactive, and the bisglycoside (II) and monoglycoside (III), which may undergo further hydrolysis (Scheme I). Figures 2 and 3 show the variation in the composition of digoxin hydrolysis mixtures as a function of time at pH 1 and 2, respectively. Each possible hydrolysis product is observed in the hydrolysates. In a series of parallel experiments, the hydrolysis of the reactive products, II and III was studied similarly.

As shown in Fig. 4, for reactions carried out at pH 1, substrate (I, II, or III) disappearance follows pseudo-first-order kinetics. The magnitude of the pseudo-first-order rate constants obtained from these graphs is shown in Table I and reflects the number of alternative pathways available for hydrolysis. These rate constants represent the sum of the individual rate constants describing the disappearance of parent species, and their magnitude increases as the number of available degradation pathways increases. Therefore, I, which can react by three alternative paths ($k_D = k_1 + k_2 + k_3 = 0.053\ min^{-1}$) has a shorter half-life (13.5 min) than II (16.5 min), which can be consumed via two pathways ($k_B = k_4 + k_5 = 0.040\ min^{-1}$); III is the most stable ($t_{1/2}$ = 24.3 min) species, since there is only one route available for its breakdown ($k_M = k_6$). Thus, differences in disappearance rates for I–III appear to reflect both probability factors



Scheme I

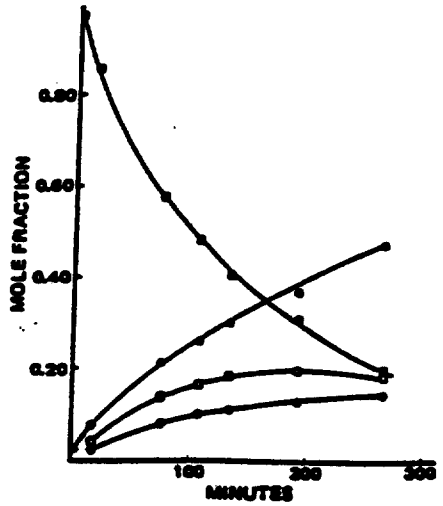


Figure 3—Time–concentration profile of digoxigenin species [I (△), II (○), III (□), and IV (△)] present in an aqueous solution of I at pH 2 incubated at 37°.

as well as variations in stereochemical restrictions imposed at specific glycosidic linkages. Over the pH range studied (1–4), disappearance of substrate showed a first-order dependence on a_{H^+} (Fig. 4). At pH 2, k_D (0.0083 min^{-1}) is one-tenth the magnitude observed at pH 1.

The digoxin hydrolysis mixture was further analyzed to determine which reaction pathways (Scheme I) were operative and to get an indication of the significance of each route. In the experiments in which digoxin was the parent, its disappearance can be described as a pseudo-first-order process, expressed by:

$$-\frac{dD}{dt} = (k_1 + k_2 + k_3)D = k_D D \qquad \text{(Eq. 1)}$$

where k_D is the sum of k_1, k_2, and k_3. Rate expressions describing the appearance of bisglycoside (B), monoglycoside (M), and genin (G) take similar forms, expressed by:

$$\frac{dB}{dt} = k_1 D - (k_4 + k_5)B \qquad \text{(Eq. 2)}$$

$$\frac{dM}{dt} = k_2 D + k_4 B - k_6 M \qquad \text{(Eq. 3)}$$

$$\frac{dG}{dt} = k_3 D + k_5 B + k_6 M \qquad \text{(Eq. 4)}$$

These differential equations can be solved[1] and the amount of each component in the mixture can be expressed as the mole fraction of all digoxin species present:

$$D/D_0 = e^{-k_D t} \qquad \text{(Eq. 5)}$$

$$\frac{B}{D_0} = Ae^{-k_D t} + \left(\frac{B_0}{D_0} - A\right)e^{-\alpha t} \qquad \text{(Eq. 6)}$$

where $A = k_4/(\alpha - k_D)$ and $\alpha = k_4 + k_5$, and:

$$\frac{M}{D_0} = A\left[Fe^{-k_D t} + \left(1 - \frac{B_0}{D_0 A}\right)Ce^{-\alpha t} - \left[F + \left(1 - \frac{B_0}{D_0 A}\right)C - \frac{M_0}{D_0 A}\right]e^{-k_6 t}\right] \qquad \text{(Eq. 7)}$$

where $F = [k_4 + (k_2/A)]/(k_6 - k_D)$ and $C = k_4/(\alpha - k_6)$, and:

$$\frac{G}{D_0} = \frac{G_0}{D_0} + \frac{1}{k_D}(k_3 + k_5 A + k_6 AF)(1 - e^{-k_D t}) + \frac{1}{\alpha}\left[k_5\left(\frac{B_0}{D_0} - A\right) + k_6 A\left(1 - \frac{B_0}{D_0 A}\right)C\right](1 - e^{-\alpha t}) + \frac{1}{k_6}\left[k_6 A\left[\frac{M_0}{D_0 A} - F - C\left(1 - \frac{B_0}{D_0 A}\right)\right]\right](1 - e^{-k_6 t}) \qquad \text{(Eq. 8)}$$

[1] Complete derivation of all equations is available from the authors on request.

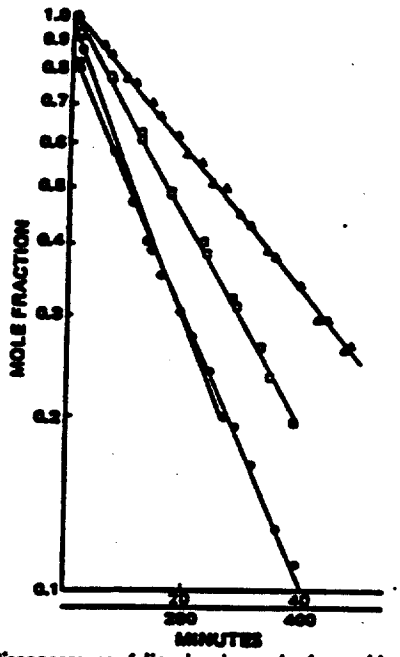


Figure 4—Disappearance of digoxigenin species from acid solution as a function of time. In the pH 1 (0–60-min time scale) solution, the parent compound was III (△), II (□), or I (○). In the pH 2 (0–600-min time scale) solution, the parent compound was I (●).

Initial conditions were defined such that at $t = 0$, the amount of component J present in the mixture was J_0.

Imposing these boundary conditions provides general expressions in which the concentration of digoxin species can be determined in samples initially containing more than one digoxigenin component. Such manipulations were necessary because samples contained small amounts of impurities formed from prior hydrolysis of the parent compound.

From the HPLC tracings obtained during hydrolysis experiments, the amount of each component in the mixture could be expressed as the mole fraction of all digoxin species present as a function of time. Expressing the composition in this form eliminates error due to variations in the HPLC injection volume. When digoxin was the parent compound, these data were fit simultaneously to Eqs. 5–8 by an adaptive simplex procedure similar to that proposed by Nelder and Mead (9).

The quality of the fit at each iteration was determined by using Eqs. 5–8 to predict concentrations for each component at each time point, calculating the difference between these theoretical concentrations and the experimentally determined levels, and then summing the squares of all differences. In this way, all six rate constants were evaluated in one fitting procedure (Table I). The accuracy of this procedure is demonstrated by comparing the pseudo-first-order rate constant (k_D), determined experimentally for digoxin disappearance, with the sum of the individual rate constants (k_1, k_2, and k_3) it represents, determined by computer fit (Table I).

Table I—Rate Constants for Digoxin Hydrolysis at pH 1 [a]

Constant	Time, min^{-1}
k_1	0.0263
k_2	0.0089
k_3	0.0193
k_4	0.0207
k_5	0.0211
k_6	0.0301
k_M [b]	0.029
$(k_4 + k_5)$ [c]	0.042
k_D [b]	0.040
$(k_1 + k_2 + k_3)$ [d]	0.0545
k_D [c]	0.053

[a] Determined by the simplex fitting procedure as described in the text. [b] Determined experimentally from pseudo-first-order plots (Fig. 4). [c] Sum equivalent to k_D. [d] Sum equivalent to k_D.

Table II—Rate Constants for Hydrolysis of II and III at pH 1

Constant	Time, min^{-1}
k_4[a]	0.0232
k_5[a]	0.0165
k_6[a]	0.0368
$k_4 + k_5$[a,b]	0.040
k_B[c]	0.040
k_M[c]	0.029
k_6[d]	0.0290

[a] Determined from the simplex fit of data obtained for II hydrolysis. [b] Sum equivalent to k_B. [c] Determined experimentally from pseudo-first-order plots of Fig. 4. [d] Determined from the simplex fit of data obtained for III hydrolysis.

In experiments where II served as a substrate, kinetics can be described by the differential Eqs. 9–11 which are integrated to give Eqs. 12–14:

$$-\frac{dB}{dt} = (k_4 + k_5)B \quad \text{(Eq. 9)}$$

$$\frac{dM}{dt} = k_5B - k_6M \quad \text{(Eq. 10)}$$

$$\frac{dG}{dt} = k_5B + k_6M \quad \text{(Eq. 11)}$$

$$\frac{B}{B_0} = e^{-\alpha t} \quad \text{(Eq. 12)}$$

$$\frac{M}{B_0} = -Ce^{-\alpha t} + \left(\frac{M_0}{B_0} + C\right)e^{-k_6 t} \quad \text{(Eq. 13)}$$

$$\frac{G}{B_0} = \frac{1}{\alpha}(k_5 - k_6C)(1 - e^{-\alpha t}) + \frac{1}{k_6}\left[k_6\left(\frac{M_0}{B_0} + C\right)\right](1 - e^{-k_6 t}) + \frac{G_0}{B_0} \quad \text{(Eq. 14)}$$

Similar treatment for III hydrolysis yields:

$$-\frac{dM}{dt} = k_6M = \frac{dG}{dt} \quad \text{(Eq. 15)}$$

$$\frac{M}{M_0} = e^{-k_6 t} \quad \text{(Eq. 16)}$$

$$\frac{G}{M_0} = \left(\frac{1}{M_0} - e^{-k_6 t}\right) \quad \text{(Eq. 17)}$$

Simplex fit of the data obtained from II and III hydrolysis to Eqs. 12–14 and 16 and 17, respectively, gave the results shown in Table II. The goodness of fit is shown by comparison of k_B and k_M, determined experimentally, with the individual rate constants [$(k_4 + k_5)$ and k_6, respectively] they represent.

These results suggest that all possible hydrolysis routes are operative and approximately equally weighted, except for the direct cleavage of two digitoxose residues from digoxin (k_5) to form III, which occurs to a lesser extent. This observation may reflect stereochemical restrictions imposed at the glycosidic linkage or could be an artifact of the computer-fitting procedure.

The results presented also suggest that the variation in digoxin bioavailability after oral administration may be explained by the pH dependence of its hydrolysis. The digoxin half-life decreased from 121 min at pH 2 to 12.5 min at pH 1. Thus, the composition and amount of digoxin species available for absorption from the stomach should vary significantly with gastric acidity. The stomach pH varies widely among individuals (pH 0.8–4) and also within a patient as influenced by emotional and physical stresses, diet, and other factors (10). Thus, the variability in oral digoxin absorption may arise from variations in gastric pH, which influence the extent to which hydrolysis occurs and effectively competes with absorption of the active drug.

EXPERIMENTAL

Kinetic Experiments—The parent compound was dissolved in aqueous solution of appropriate pH and adjusted to an ionic strength of 1.0 with solid potassium chloride. The final concentration of substrate in all solutions was approximately 10 γ/ml. Ten milliliters of solution was incubated at 37°. At timed intervals, 25-μl samples were withdrawn[2] and subjected directly to HPLC analysis.

HPLC Analysis—Chromatography was performed on a component system[3]; the eluent was monitored spectrophotometrically at 225 nm. The separation utilized a column[4] (4 mm × 30 cm) operating at 2.0 ml/min with methanol–water (55:45) as the mobile phase. The retention volumes for IV, III, II, and I were 4.4, 4.2, 8.2, and 12.8 ml, respectively. The practical detection limit for digoxin was 25 ng from 25-λ injections. Components were quantitated by measuring peak heights and comparing the height with an external standard containing a mixture of known amounts of the four components.

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[2] Hamilton syringes.

[3] The chromatographic system consisted of a Waters M6000A solvent delivery system and a U6K universal injector coupled with a Varian Vari-Chrom detector.

[4] μBondapak C_{18}, Waters Associates, Inc.

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PHARMACEUTICA ACTA HELVETIAE

Photostability studies of ouabain, α-acetyldigoxin and digoxin in solid state *

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Abstract

Ouabain, α-acetyldigoxin and digoxin were subjected to irradiation using different light sources in crystalline state and their respective yields of photoproducts were determined densitometrically. α-Acetyldigoxin was found to be less stable than digoxin yielding a higher percentage of photoproducts under each light source examined. Ouabain showed photostability under the conditions of investigation.

Key words: α-Acetyldigoxin; Digoxin; Ouabain; Photostability; UV-light

1. Introduction

Cardiac glycosides are some of the early plant products to be employed in clinical practice. Their unique pharmacological activities, regardless of the risk of their acute and cumulative toxicity, made them to be the drugs of choice in various ailments of heart (Thomas et al., 1990). They act on the heart by an array of direct and indirect effects, the result of which is to increase the force of contraction (positive inotropic effect) and slow the rate of atrioventricular (AV) conduction. There have been major advances in recent years in our understanding of how these drugs act and how to best use them clinically (Thomas, 1981).

As part of our ongoing research programme, we have been engaged in the investigation of photostability of steroidal drug substances (Zappel, 1993). We have found that sometimes pharmacopoeial recommendation requires protection from light irradiation although the substance is stable against light, or there is no light protection recommended when the drug is not stable. In this connection we checked the accuracy of the given storage conditions for different steroidal drugs.

We found interesting photoproducts of isomerisation (Ekiz-Gücer and Reisch, 1991) and dimerisation (Reisch et al., 1992, 1993) with corticosteroidal and sex steroidal drugs, and isolated respective products in their pure form and characterized whenever possible.

As an extension of the steroidal photochemistry work, three cardiac glycosides – ouabain, α-acetyldigoxin and digoxin – were selected and their photostability studies have been carried out and the results are presented in this communication (see Fig. 1 for the structures).

A general indication to protect these drugs from light has been mentioned in several pharmacopoeias (Table 1). The monograph of α-acetyldigoxin has been discontinued in many of the recent editions and for digoxin and ouabain precautions have been mentioned to protect the drug in well-closed containers.

* Photochemical Studies Part 67 (Reisch and Abdel-Khalek, 1993).

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Table 1
Pharmacopoeial requirement for light protection

Compound	EP(M)	USP	PH	PB	BP	EP	DAB
Ouabain	+	/	+	+	/	/	+
Digoxin	+	+	+	+	+	+	+
α-Acetyldigoxin	−	/	/	/	/	/	/

EP(M): Extra Pharmacopoeia (Martindale) 1993(30); USP: United States Pharmacopoeia 1990(22); PH: Pharmacopoeia Helvetica 1991(7); PB: Pharmacopoeia Belgica 1982(6); BP: British Pharmacopoeia 1988(14); EP: European Pharmacopoeia 1980(2); DAB: Deutsches Arzneibuch 10 (1991).
+: light protection; −: no light protection; /: not registered.

2. Experimental procedures

2.1. Chemicals

Ouabain (Merck/Darmstadt), α-Acetyldigoxin (Sandoz AG), Digoxin (Boehringer/Mannheim), Aerosil 200 (Degussa). These glycosides were not further purified. The solvents (Baker) used were of analytical grade.

2.2. Equipment

The irradiation was carried out using a rotating reactor (Grāntzel/Karlsruhe), with a low-pressure mercury-lamp (254 nm) or day-light source (Takács et al., 1990). An irradiation chamber (Heraeus/Hanau) with a high-pressure mercury-lamp (Q 300) was used for on-plate and petri dish methods. The densitometric determinations were made using a chromatogram spectrophotometer KM 3 (Zeiss/Oberkochem).

2.3. Thin layer chromatography (TLC)

Prepared TLC-plates, Silica gel 60 F_{254} (precoated, aluminium sheets, 0.2 mm thickness, Merck 5549) were used for analytical TLC and 60 F_{254} (glass plates, 0.25 mm thickness, Merck 5715) for densitometric work. Solvent system I: CH_2Cl_2/MeOH (97:3); II: CH_2Cl_2/MeOH (95:5); III: CH_2Cl_2/MeOH (7:3). Detections for α-acetyldigoxin and digoxin were made by spraying with ethanolic sulfuric acid (1:1) and heating at 110°C

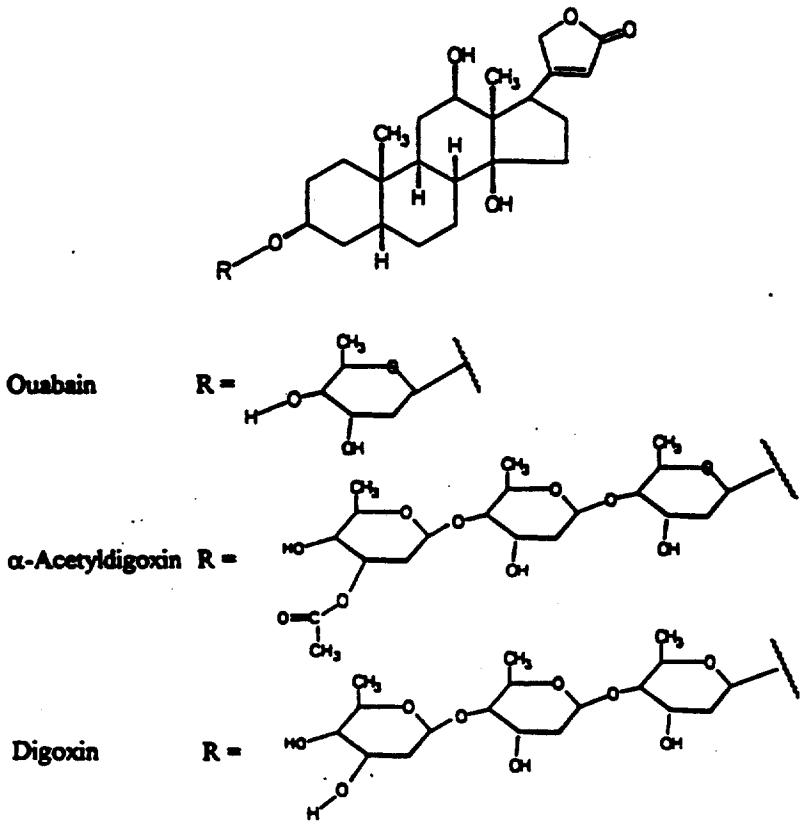

Fig. 1. Structural formulas of ouabain, α-acetyldigoxin and digoxin.

for 10 min. For ouabain detection was done by UV-light at 254 nm.

2.4. General procedure

Rotating reactor

The drug (1 g) and 20% Aerosil 200 (0.2 g) were thoroughly mixed using the gradient mixing technique, taken in a quartz glass tube and fixed to a rotating reactor. The details of the construction of the reactor have been given in our previous report (Takács et al., 1991). Dry nitrogen or synthetic air was circulated through continuously. The irradiation was carried out for 96 h, taking samples intermittently from the mixture for every 24 h.

Irradiation chamber

The methods employed were the irradiation on a plate and in petri dishes as described earlier from these laboratories (Reisch and Topaloglu, 1986).

Densitometric determination:

The detailed method has been described in our earlier publication (Ekiz-Gücer et al., 1991).

3. Results and discussion

In studies using different irradiation methods it has been observed that ouabain is relatively less sensitive to day-light and uv radiation than the other compounds as the maximum yield of photoproducts was determined to be 7.5% after 96 h of low-pressure mercury lamp irradiation under synthetic air atmosphere (Table 2).

Digoxin yielded a lower percentage of photoproducts than did α-acetyldigoxin in all five methods of irradiation employed.

Digoxin showed two main products on irradiation. There were no differences when the IR-, ^{1}H NMR- and ^{13}C NMR spectral data of the first product were compared with those of the starting material. Since the M^+-peak appeared at m/e = 762 it is 18 mass units less than the starting material. It is possible that the H_2O is cleaved from the sugar moiety because a cleavage of H_2O from the steroidal moiety would have been indicated clearly by NMR spectrum.

The signals for 20 H and 22 H and the signals for the carbon atoms 20, 22 and 23 of the second main photoproduct disappeared in the ^{1}H NMR and in the ^{13}C NMR spectrum, respectively. The lactonic carbonyl absorption disappeared in the IR spectra. These results show that the α,β-unsaturated lactone ring of the cardenolide is decomposed or cleaved (Penelope et al., 1980). Further we noticed through our previous work on digitoxin that this lactone ring of the molecule is very reactive (Ekiz-Gücer and Reisch, 1991). A peak beyond m/e = 500 was observed neither through CI nor through SIMS and therefore it was not possible to obtain the molecular peak of the product for complete characterisation.

Table 2
Yield of photoproducts in %

Compound	Irradiation method	24 h	48 h	72 h	96 h
Ouabain	A	3.7	5.3	6.4	7.5
	B	2.2	4.5	5.9	6.1
	C	0.8	1.3	1.7	2.1
	D	0.0	0.0	0.2	0.3
	E	0.0			
Digoxin	A	6.7	15.6	26.1	27.8
	B	3.2	12.7	25.7	26.9
	C	3.8	5.6	9.7	11.1
	D	0.0	0.9	1.2	1.6
	E	36.8			
α-Acetyldigoxin	A	7.8	18.4	29.2	33.4
	B	9.2	20.9	30.7	36.9
	C	4.3	6.7	10.3	13.2
	D	0.0	1.2	1.5	2.4
	E	32.6			

A: Low-pressure mercury lamp (synthetic air); B: Low-pressure mercury lamp (nitrogen); C: High-pressure mercury lamp; D: Daylight source; E: On plate method.

α-Acetyldigoxin yielded 37% of photoproducts on irradiation with a low-pressure mercury lamp under nitrogen atmosphere (Table 2). Although a mixture of two photoproducts (120 mg) was isolated, their very close R_f values in a variety of solvent systems made it impossible to separate them. Finally attempts to separate the two photoproducts using medium-pressure prepacked columns, a series of eluants and different pressures were also unsuccessful. But there was no change of the spectral data of the mixture other than an additional peak at δ = 176 ppm in the ^{13}C NMR spectrum in comparison to the starting material. However, the important result is that α-acetyldigoxin must be stored under light protection conditions as it is photounstable, although this is not mentioned in the *Extra Pharmacopoeia (Martindale)*.

Ouabain showed only a little decomposition (Table 2). Therefore this compound can be labelled as photostable under normal pharmaceutical storage conditions although most of the pharmacopoeias indicate that it is photounstable (Table 1).

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AQUATIC TOXICOLOGY

A comparison of the toxicity of 50 reference chemicals to freshly isolated rainbow trout hepatocytes and *Daphnia magna*

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Abstract

There is a need for rapid and cost-effective in vitro tests or test batteries in aquatic toxicology which could be used as tools in evaluating the toxicity of chemicals. In the present study the toxicity of 50 reference chemicals was evaluated by determining immobility in *Daphnia magna* (24 h incubation) and ^{86}Rb-leakage in freshly isolated rainbow trout hepatocytes (3 h incubation). Regression analysis of the data on the EC_{50} for *Daphnia* and EC_{50} for the rubidium leakage in hepatocytes showed a correlation of 0.71 ($p < 0.0001$). The slope of the regression line (0.68) differed markedly from a 1:1 correlation. *Daphnia* was more sensitive to the chemicals than hepatocytes and the discrepancy between the two tests increased with increasing toxicity of the chemicals. A comparison of the data from the hepatocyte test with published data on the toxicity of the reference chemicals to cultured mammalian hepatocytes (24 h incubation), indicated that the lower sensitivity of the fish hepatocytes compared to *Daphnia* is not due to the shorter incubation time used in the hepatocyte test. A comparison of the data from the *Daphnia* test with published data on the toxicity of the reference chemicals to various mammalian cells, showed a similar discrepancy between the *Daphnia* test and the cellular tests as in the case of the comparison between the *Daphnia* test and the rainbow trout hepatocyte test, indicating that the *Daphnia* test in general is more sensitive than conventional cellular cytotoxicity tests. It is concluded that further studies, using more sensitive endpoints than rubidium leakage, are needed before it can be decided whether or not freshly isolated rainbow trout hepatocytes are suitable in routine toxicity testing.

Key words: Hepatocyte; Rainbow trout; *Daphnia magna*; Cytotoxicity; MEIC chemicals

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1. Introduction

In mammalian toxicology, much effort has been made during the last decade to develop and evaluate in vitro tests which could be used to predict the toxicity of chemicals to man, and several projects with the aim to validate the predictability of in vitro tests have been started (Ekwall et al., 1991). One of the most comprehensive evaluation projects is the 'Multicenter Evaluation of In Vitro Cytotoxicity' (MEIC) programme. The ultimate goal of this project is to select batteries of in vitro tests which could be used as supplements or alternatives to animal tests (Bondesson et al., 1989). Fifty reference chemicals have been selected by an organization independent of the MEIC project, and laboratories willing to participate in the project are invited to test the chemicals in their own in vitro systems and submit the results to the MEIC project for evaluation. The majority of the MEIC reference chemicals have now been tested in more than 50 different in vitro systems (Ekwall et al., 1992).

The basic assumption underlying the expectation that in vitro tests can be used to predict the toxicity of chemicals to man, is that most of the toxic chemicals exert their effect by interacting with basic cellular functions common to all human and mammalian cells (Ekwall, 1983). The cytotoxic concentration established in vitro should thus predict the toxic blood or tissue concentration (Ekwall, 1983). If the cytotoxic data are combined with a model for prediction of toxicokinetics (absorption, distribution, biotransformation, and excretion) of the chemical, the acute oral toxicity of the chemical can be predicted.

One important aim in aquatic toxicology would be to develop test batteries which could be used to predict the concentrations where chemicals exert adverse effects on the cells of the organisms living in aquatic ecosystems. Assuming that most chemicals exert their effect by interfering with processes common for most cells (Ekwall, 1983), the number of taxa used in a test battery can be reduced. Toxicity data obtained can then be combined with models predicting the behavior of a chemical in the aqueous phase and with models on the toxicokinetics of the chemical. Such a putative test battery would increase our possibility to predict adverse effects of chemicals in the aquatic environment.

The present study was undertaken in a search for suitable in vitro tests which could be included in an in vitro test battery for toxicity testing in aquatic toxicology. Such a putative test battery should, in our opinion, include cells from vertebrates. In the present study we have focused on hepatocytes isolated from rainbow trout. Hepatocytes are frequently used in mammalian toxicological research but even though hepatocytes have been successfully isolated from several species of fish for many years, they have not yet been generally used in toxicity testing. Their usefulness as model cells in toxicity testing has consequently not been adequately explored. In evaluating the suitability of fish hepatocytes in toxicity testing we have used the 50 MEIC reference chemicals and tested these chemicals on both freshly isolated rainbow trout hepatocytes and *Daphnia magna*, and in analyzing and evaluating the data we have also utilized data accumulated within the MEIC project on mammalian cells.

2. Material and methods

Chemicals

The test chemicals used in this study are the reference chemicals used by the Multicenter Evaluation of In Vitro Cytotoxicity (MEIC) programme (Bondesson, et al. 1989). All the chemicals used, except malathion, were commercial chemicals of reagent grade. Malathion, with a purity of 95%, was a gift from Kemira OY (Vasa, Finland). The chemicals were dissolved as indicated in Table 1. Those chemicals which required methanol or ethanol as solvents were dissolved up to their solubility limits, in order to minimize the amount of solvent in the test samples. At the highest concentration of solvents used, no increase in lethality or rubidium leakage was observed. ^{86}Rb (RbCl in aqueous solution) was obtained from Amersham (Buckinghamshire, UK).

Daphnia magna

Daphnia magna were obtained from the Department of Biology, University of Turku. The daphnia were cultured in M4 media (Elendt and Bins, 1990) at a temperature of 21 ± 1°C with a photoperiod of 12 h light and 12 h dark. They were fed *Scenedesmus* algae every second or third day depending on the density of the culture.

Acute toxicity tests were performed in Standard Reference Water (SRW; $NaHCO_3$ 2.4 mM, K_2SO_4 0.15 mM, $CaCl_2$ 2.0 mM, KH_2PO_4 0.01 mM, pH 7.6) according to the OECD standard protocol (1980) with a few modifications. The tests were performed at 21 ± 1°C in 100 ml polystyrene vessels, with 50 ml liquid in each. Twenty neonates (<24 h old) were introduced into vessels containing different concentrations of the test chemical and the vessels were closed with a polyethene cap. There was no feeding and no aeration during the tests. Immobilization was determined visually after 3 and 24 h. For each chemical the test was repeated at least three times.

Rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) with a mean body weight of 370 g were obtained from a commercial fish farm. They were kept in dechlorinated tap water in polypropylene aquaria at 15°C with constant aeration and filtration of the water for at least one week prior to the experiments. The photoperiod was 12 h light and 12 h dark and they were not fed.

Hepatocyte isolation

The fish were stunned with a sharp blow to the base of the skull. The hepatocytes were isolated by a two-step collagenase perfusion originally described by Seglen (1976) with a slight modification of the procedure (Råbergh et al, 1992). In short, the liver was perfused by cannulating the portal vein. The liver was first perfused with 250 ml of a Ca^{2+}-free buffer containing 142 mM NaCl, HEPES 10 mM, pH 7.4, EGTA 0.5 mM, and 0.2% bovine serum albumin (BSA). This buffer weakens the calcium dependent adhesion factors between the cells. The liver was cleared from blood and removed from the fish. The buffer was then changed to a collagenase buffer containing 66.7 mM NaCl, 6.7 mM KCl, 4.8 mM $CaCl_2$, 100 mM HEPES, pH 7.6, 0.5% BSA

0.5% and 0.05% collagenase. This buffer was recirculated and the perfusion continued for 10 to 15 min. The liver was then flushed with an incubation buffer containing 68 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 1.1 mM KH_2PO_4, 0.7 mM Na_2SO_4, 30 mM HEPES, 30 mM TES, 36 mM Tricine, pH 7.6, 1% BSA and 10 mM glucose. The gallbladder was removed and the liver was mechanically disintegrated. The suspension was then filtered through nylon filters (250 and 70 μm) and the cells were collected in incubation buffer. Following a 15 to 20 min incubation at 15°C, the cells were washed twice (10 min at 200 × g) in order to remove dead cells. The cell density was adjusted to 2×10^7 cells per ml. The viability of the cells was routinely tested by trypan blue exclusion and it exceeded 90%. Following isolation the hepatocytes were stored on ice and experiments were finished within 5 h after isolation.

Rubidium leakage

The cells were loaded with $^{86}Rb^+$ (185 kBq/ml) at 15°C for 1 h in the incubation buffer. After the incubation with $^{86}Rb^+$, the cells were washed 6 times (1 min, 500 × g) with ice cold incubation buffer. In the two last washes, BSA was omitted from the buffer. The viability following the washing was routinely tested and it exceeded 95%. The presence of BSA in the washing buffer was essential for a high viability of the cell suspension but to avoid an interaction of BSA with the test chemicals, BSA was omitted in the two last washes. Following the washing, the cell density was readjusted to 2×10^7 cells per ml.

The toxicity tests were performed in 6 ml plastic scintillations vials; 0.1 ml cell suspension was added to 0.9 ml buffer (without BSA) containing different concentrations of the test chemicals. The final cell density was 2×10^6 cells per ml. The samples were incubated for 3 h at 15°C in a shaking bath. Following the incubation, the samples were centrifuged at 5000 × g for 1 min, and aliquots of the supernatants were taken for the determination of radioactivity. Radioactivity was determined by using a liquid scintillation counter and Optiphuse 'HiSafe' 3 (LKB, Loughborough, Lecis, England) as a scintillation fluid. Rubidium leakage is expressed as percentage of tracer released in relation to the total ^{86}Rb-content, which was determined by lysing the cells with Triton-X 100 and freezing. Immediately before the leakage studies were started, supernatant samples of the washed hepatocyte suspension were collected by centrifugation in order to determine extracellular radioactivity. The extracellular radioactivity was subtracted from the experimental values when calculating the amount of tracer released. With each test chemical the experiments were repeated at least three times.

Calculation of EC_{50}

For each chemical, five concentrations were used for the determination of EC_{50}s. EC_{50} values were calculated using regression analysis after linearisation of the dose/response curves by logarithmic transformation of the concentrations (statistical program: GraphPad 4).

Table 1
Twenty-four hour EC_{50} values (mean ± S.D.) for *D. magna* and 3 h EC_{50} values (mean ± S.D.) for rainbow trout hepatocytes

Chemical	Molecular weight	*D. magna* EC_{50} (mM)	Hepatocyte EC_{50} (mM)
1. Acetaminophen	151.16	•	•
2. Aspirin[B]	180.15	8.15 ± 0.91	27.6 ± 1.02
3. Ferrous sulfate	151.91	0.094 ± 0.09	0.008 ± 0.00082
4. Diazepam[A,B]	284.76	0.015 ± 0.0013	2.314 ± 0.373
5. Amitriptyline[A]	277.39	$(4.15 \pm 1.06) \times 10^{-1}$	0.475 ± 0.045
6. Digoxin[A,C]	780.92	0.031 ± 0.001	•
7. Ethylene glycol	62.07	782.7 ± 77.9	6790 ± 1451
8. Methyl alcohol	32.04	649.3 ± 48.0	5364 ± 2396
9. Ethyl alcohol	46.07	297.7 ± 43.6	1724 ± 265
10. Isopropyl alcohol	60.09	114.0 ± 7.0	1327 ± 220
11. 1,1,1-Trichloroethane	133.42	•	86.5 ± 11.2
12. Phenol	94.11	0.097 ± 0.015	23.4 ± 0.29
13. Sodium chloride	58.45	37.37 ± 16.24	304.9 ± 51.7
14. Sodium fluoride	42.00	7.23 ± 0.151	20.8 ± 15.2
15. Malathion	330.35	$(1.07 \pm 0.11) \times 10^{-1}$	1159 ± 146.2
16. 2,4-Dichlorophenoxy acetic acid[A,B]	221.04	1.124 ± 0.084	19.45 ± 1.98
17. Xylene[B]	106.16	•	•
18. Nicotine	162.23	0.035 ± 0.015	44.7 ± 1.3
19. Potassium cyanide[A]	65.11	$(9.35 \pm 2.80) \times 10^{-1}$	65.3 ± 8.3
20. Lithium sulfate[A]	109.88	1.79 ± 0.43	972 ± 114
21. Theophylline	180.07	0.861 ± 0.04	•
22. Dextropropoxyphene HCl	261.14	$(55.76 \pm 49.2) \times 10^{-3}$	2.86 ± 0.11
23. Propranolol HCl[A]	259.34	$(10.4 \pm 4.0) \times 10^{-4}$	1.86 ± 1.56
24. Phenobarbital[B]	232.23	•	29.2 ± 3.6
25. Paraquat[A]	186.25	$(62.7 \pm 9.0) \times 10^{-3}$	???
26. Arsenic trioxide	197.82	•	•
27. Cupric sulfate[A]	159.61	$(1.16 \pm 0.13) \times 10^{-1}$	0.281 ± 0.249
28. Mercuric chloride[A]	271.52	$(0.011 \pm 0.003) \times 10^{-1}$	$(0.164 \pm 0.045) \times 10^{-1}$
29. Chlordiazine HCl[A,B]	370.56	$(1.87 \pm 0.3) \times 10^{-3}$	0.044 ± 0.009
30. Thallium sulfate[A]	504.85	$(16 \pm 1.6) \times 10^{-1}$	•
31. Warfarin[A]	308.34	0.288 ± 0.007	•
32. Lindane[A,B]	290.85	0.05 ± 0.006	1.19 ± 0.11
33. Chloroform[B,A]	119.38	4.80 ± 0.185	28.04 ± 3.12
34. Carbon tetrachloride[B,A]	153.84	0.484 ± 0.036	14.32 ± 2.03
35. Isoniazid	137.14	0.617 ± 0.088	•
36. Dichloromethane[B,A]	84.93	22.86 ± 0.85	111.3 ± 4.8
37. Barium nitrate[A]	261.38	0.512 ± 0.028	•
38. Hexachlorophene[A,B]	406.91	$(0.487 \pm 0.024) \times 10^{-1}$	0.029 ± 0.005
39. Pentachlorophenol[B,B]	266.34	$(3.22 \pm 0.3) \times 10^{-1}$	0.186 ± 0.06
40. Verapamil HCl[B,A]	491.05	0.665 ± 0.008	3.75 ± 0.33
41. Chloroquine phosphate[B,A]	515.90	0.096 ± 0.011	40.66 ± 20.58
42. Orphenadrine HCl[B,A]	305.80	0.029 ± 0.005	0.938 ± 0.283
43. Quinidine sulfate[B,A]	746.95	0.060 ± 0.002	2.10 ± 0.52
44. Diphenylhydantoin[A]	252.27	•	5.63 ± 2.33
45. Chloramphenicol[B,A]	323.13	1.68 ± 0.082	0.011 ± 0.001
46. Sodium oxalate[B,A]	134.00	2.97 ± 0.097	1.49 ± 0.302

Table 1 (continued)

Chemical	Molecular weight	*D. magna* EC'_{50} (mM)	Hepatocyte EC'_{50} (mM)
47 Amphetamine sulfate[a,h]	368.50	0.164 ± 0.02	53.05 ± 8.26
48 Caffeine[a,h]	194.19	3.521 ± 0.394	172.5 ± 97.8
49 Atropine sulfate[a,h]	694.85	0.372 ± 0.038	16.83 ± 3.31
50 Potassium chloride[a,h]	74.55	15.12 ± 0.152	277.4 ± 113.3

[a]Immobilization and/or rubidium leakage was below 50% at the solubility limit of the chemical in the incubation buffer.
[b]Dissolved in H_2O (*D. magna*).
[c]Dissolved in ethanol (*D. magna*).
[d]Dissolved in methanol (*D. magna*).
[e]Dissolved in H_2O (hepatocytes).
[f]Dissolved in ethanol (hepatocytes).
[g]Dissolved in methanol (hepatocytes).

3. Results

Comparison between the 3 h hepatocyte test and the 24 h Daphnia test

The EC'_{50} values for rubidium leakage in rainbow trout hepatocytes and the 24 h EC_{50} values for *Daphnia* are listed in Table 1. In the hepatocyte test there were nine chemicals for which the rubidium leakage was below 50% and in the *Daphnia* test there were six chemicals where the immobility was below 50% at the solubility limit of chemicals in the test medium, but only three of the chemicals where the same in both tests (Table 1). In the 3 h test with *Daphnia*, only six chemicals (ethanol, methanol, ethylene glycol, 2,4-dichlorophenoxyacetic acid and potassium chloride) caused an immobility exceeding 50% at concentrations which caused 100% immobility following 24 h exposure and for most of the other chemicals no immobility was observed following 3 h exposure (results not shown). Thus there is a pronounced discrepancy between the results from the 3 h and the 24 h *Daphnia* test. A 3 h exposure time is definitely too short in the case of the *Daphnia* test.

In Fig. 1, the 3 h hepatocyte test is compared to the 24 h *Daphnia* test. The correlation coefficient (38 chemicals) is 0.71 ($p < 0.0001$). As can be seen, however, the slope of the regression line differs markedly from a 1:1 correlation. The *Daphnia* test is more sensitive than the hepatocyte test and the discrepancy between the tests increases with increasing toxicity of the chemicals, the slope of the regression line being 0.68 (Fig. 1). Only three chemicals, iron sulphate (3), chloramphenicol (45) and sodium oxalate (46), are more toxic to the hepatocytes than to *Daphnia* (the values are below the theoretical line for 1:1 correlation). The most distinct outliers from the regression line (deviate more than two orders of magnitude from the regression line) are iron sulphate (3), malathion (15) and chloramphenicol (45). The reason to the marked deviation from the regression line is in the case of malathion likely due to the fact that malathion is an acetylcholine esterase inhibitor and should consequently be more toxic to an intact organism than to an isolated cell. Furthermore, malathion is more

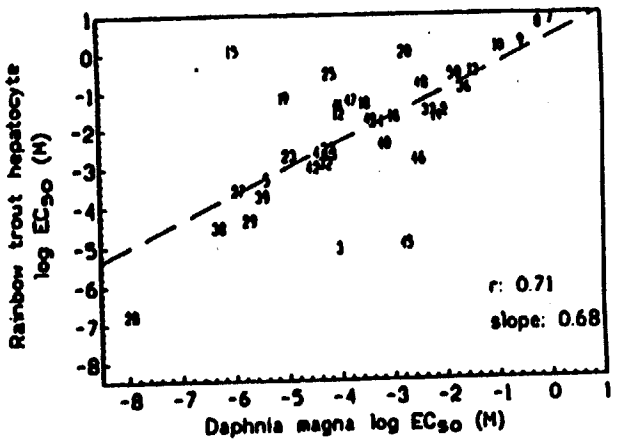

Fig. 1. A comparison between EC'_{50} for rubidium leakage in isolated rainbow trout hepatocytes and EC_{50} for immobilization in *Daphnia magna* for the MEIC reference chemicals (38 chemicals). The hepatocytes were incubated with the chemicals for 3 h at 15°C and *D. magna* for 24 h at 20°C. The numbers refer to the numbering in the MEIC list (Table 1).

rapidly metabolized in hepatocytes than in *Daphnia*, because the carboxyesterase-catalysed hydrolysis of malathion is more efficient in higher animals than in insects (Norton, 1975) and possibly also in other arthropods. Ferrous salts have been shown to be hepatotoxic, possibly due to iron-induced lipid peroxidation in hepatic mitochondria and microsomes (Bacon et al., 1986). This may explain the higher sensitivity of hepatocytes to iron sulphate. In the case of chloramphenicol we do not have any reasonable explanation to the higher sensitivity of hepatocytes. Chloramphenicol is an antimicrobial compound which inhibits protein synthesis in bacteria by binding to the 50S ribosome subunit. It inhibits protein synthesis to some extent also in eukaryotic cells, especially the protein synthesis in mitochondria which have a ribosome subunit which resembles that of the bacterial 50S subunit (Sande and Mandell, 1980).

Comparison between freshly isolated rainbow trout hepatocytes and mammalian primary hepatocyte cultures

A reasonable explanation to the lower sensitivity of the 3 h hepatocyte test in comparison to the 24 h *Daphnia* test would be the much shorter incubation time used with the hepatocytes. No data on the toxicity of the MEIC reference chemicals to fish hepatocytes in primary cultures following long-term studies have been published. Data for a comparison between short-term studies on freshly isolated hepatocytes and long-term studies on cultured fish hepatocytes are therefore not available. However, several laboratories have tested the MEIC reference chemicals in primary cultures of mammalian hepatocytes and data on the first 30 MEIC chemicals following

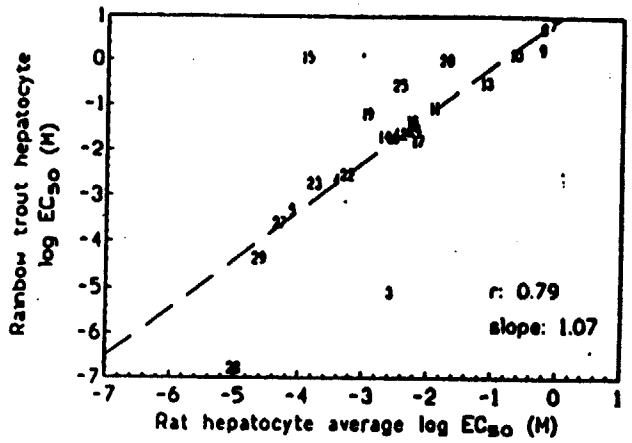

Fig. 2. A comparison between EC_{50} for rubidium leakage in isolated rainbow trout hepatocytes following 3 h incubation and EC_{50} for various endpoints in mammalian primary hepatocyte cultures following 21–24 h incubation for the first 30 MEIC reference chemicals (average of four studies; from Ekwall et al., 1992). The numbers refer to the numbering in the MEIC list (Table 1).

21–24 h exposure have been published (Ekwall et al., 1992). A comparison of our data with those from studies carried out with primary cultures of hepatocytes could be helpful in deciding whether a 3 h exposure is too short. Fig. 2 shows a comparison between our data and mammalian 21–24 h tests with primary cultures (average of five studies). As seen in Fig. 2, the correlation is rather high ($r = 0.79$, $P < 0.0001$) and the deviation of the regression line from a 1:1 correlation is small. Freshly isolated rainbow trout hepatocytes appear to be slightly less sensitive than mammalian hepatocytes in primary cultures and the difference in sensitivity is, contrary to which was the case in the comparison with *Daphnia*, slightly more pronounced with less toxic chemicals (slope 1.07). The discrepancy between the 3 h hepatocyte test and the 24 h *Daphnia* test can thus not be attributed solely to the shorter incubation time used in the hepatocyte test.

A comparison between the Daphnia test and tests made with animal cell lines

Daphnia is generally considered to be rather sensitive to various types of toxicants. It is possible that the discrepancy between the *Daphnia* test and the hepatocyte test found in the present study is not exclusive for hepatocytes, but may be valid for other tests based on cells from higher animals as well. We therefore compared our data from the *Daphnia* test on the first 30 MEIC chemicals with data from tests utilizing established mammalian cell lines (average of 14 studies; Ekwall et al., 1992). As can be seen in Fig. 3, the deviation of the regression line from a 1:1 correlation is very similar (slope 0.63) to that found in the case of freshly isolated fish hepatocytes

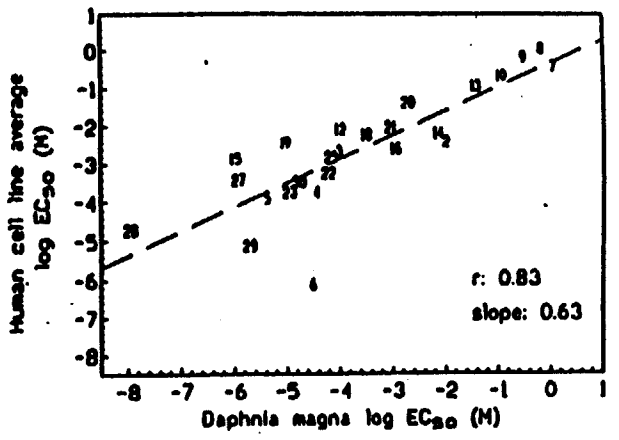

Fig. 3. A comparison between EC_{50} for various endpoints in established mammalian cell lines (average of 14 studies, from Ekwall et al., 1992) and EC_{50} for immobilization in *D. magna* for the first 30 MEIC reference chemicals. The numbers refer to the numbering in the MEIC list (Table 1).

(Fig. 1). Thus, *Daphnia* in general seems to be more sensitive to toxicants than cells from higher animals.

Daphnia and hepatocyte tests and acute toxicity in fish

Unfortunately there are very little data on the acute toxicity of the MEIC reference chemicals to fish. The data available are very inhomogeneous, because the toxicity tests on fish have been carried out by different laboratories which have used different species of fish and different exposure times. For an optimal comparison, the toxicity tests should have been carried out by the same laboratory using the same protocol for all the chemicals. Nevertheless, we have compared our data from the *Daphnia* and the hepatocyte tests with literature data on the acute toxicity of the MEIC reference chemicals to fish (Kemikalieinspektionen, 1989; Nikunen et al., 1990). In selecting the data, preference has been given to toxicity data on rainbow trout. If no data on rainbow trout were found, data from tests carried out with fathead minnow (*Pimephales promelas*), guppy (*Poecilia reticulata*) and bluegill sunfish (*Lepomis macrochirus*) were chosen. In Fig. 4, fish LC_{50} data have been compared with data from the *Daphnia* test (18 chemicals). The correlation coefficient is high (0.91, $p < 0.0001$) and the deviation of the regression line from a 1:1 correlation is very small, indicating a very similar sensitivity of fish and *Daphnia* to the tested chemicals. Fig. 5 shows a comparison between fish LC_{50} values and the 3 h hepatocyte test (20 chemicals). The correlation coefficient is 0.73 ($p < 0.0003$) and the slope of the regression line is 0.58. The deviation of the regression line from a 1:1 correlation is rather similar to that in the comparison of hepatocytes with *Daphnia* (Fig. 1).

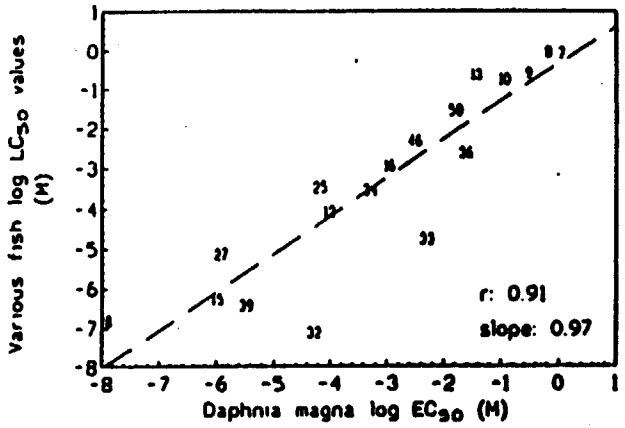

Fig. 4. A comparison between LC_{50} for fish (literature data) and EC_{50} for immobilization in *D. magna* for the MEIC reference chemicals (18 chemicals). The numbers refer to the numbering in the MEIC list (Table 1).

4. Discussion

The difficulties in translating or applying results obtained in single-species tests or in small-scale laboratory tests to realistic field conditions have been repeatedly discussed among aquatic ecotoxicologists (see for example Lee and Jones, 1983;

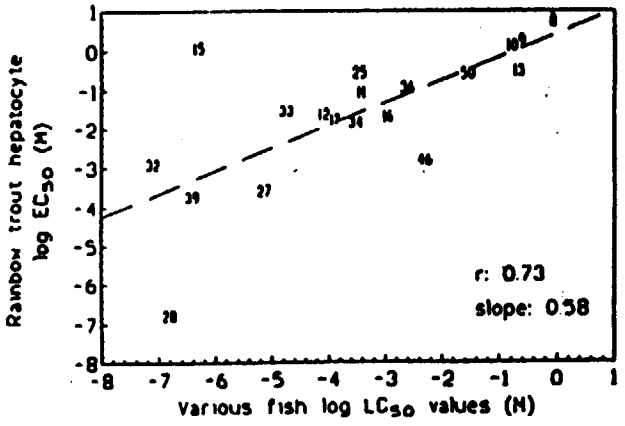

Fig. 5. A comparison between LC_{50} for fish (literature data) and EC_{50} for rubidium leakage in isolated rainbow trout hepatocytes for the MEIC reference chemicals (20 chemicals). The numbers refer to the numbering in the MEIC list (Table 1).

Leppäkoski and Bondsdorff 1989). One of the main problems is that there are very little data available on the ability of small-scale laboratory tests to predict the environmental effects of a chemical. In the projects aiming to evaluate the reliability of in vitro tests in mammalian toxicology (Ekwall et al., 1991), the in vitro toxicity data are validated using rodent or human toxicity data. A similar validation process is not possible in aquatic toxicology, unless the chemical is tested in very complicated mesocosms. This would be very time consuming and expensive and is not possible for regulatory purposes. One possible way out from this dilemma would be to develop a test battery which could be used to define the concentration where a chemical exerts adverse effects on the cells of the organisms living in aquatic ecosystems. If the in vitro toxicity data obtained were combined with models predicting the behavior of the chemical in the aqueous phase and with models predicting the toxicokinetics of the chemical, such a putative test battery would increase our possibility to predict adverse effects of chemicals in the aquatic environment. Assuming that most toxic chemicals exert their effect interfering with processes common for many types of cells (Ekwall, 1983), the selection of cells or organisms to be included in the test battery, could be based on functional aspects and the number of taxa necessary to be included could thus be reasonable.

A lot of different in vitro tests including bacteria, protozoans, phytoplankton and aquatic invertebrates have been used in the assessment of adverse effects of chemicals to aquatic organisms (for review, see Isoman et al., 1994) and many tests, especially tests using invertebrates, have been standardized for routine toxicity testing. One of the most frequently used test organisms is *Daphnia magna*. *Daphnia* is generally considered to be quite sensitive to different toxicants, and a highly significant correlation between acute *Daphnia* and fish toxicity has been demonstrated (Kaiser and Esterby, 1991). Cells isolated from aquatic organisms have not yet been generally used in toxicity testing, but during the last decade a rapidly growing interest in the use of fish cells in toxicity testing can be noticed. In aquatic in vitro toxicology there are, however, so far rather few studies with a comparative approach and few studies attempting to evaluate the predictability of the tests. The toxicity tests are often carried out with a limited set of chemicals and the chemicals tested have often been structurally related. Although the MEIC chemical list includes many chemicals which are of very little interest in aquatic toxicology, there is an obvious advantage in using these reference chemicals because the chemicals included are 'unselected' and very heterogenous and should therefore represent a broad spectrum of toxic mechanisms. Whether or not the chemicals are relevant in aquatic environments is of secondary importance in the context. Furthermore, a lot of toxicity data on the MEIC chemicals from different in vitro systems, also from non-mammalian systems, have already been published (Ekwall et al. 1992) and this enables valuable comparative considerations.

In a search for candidates for a place in a putative test battery in aquatic toxicology, it is evident that the possibility to use fish cells in such a test battery should be explored. It seems possible that toxicity tests with fish cells could partly replace or supplement the rather expensive acute toxicity tests with fish. Cellular tests could also contribute to a understanding of the toxic mechanisms involved, because a proper choice of endpoints could provide information on sublethal effects and toxic mecha-

nisms. One of the first cells to choose would be the liver cell, because the liver may be the target organ for many toxicants and the liver plays an important role in the biotransformation of xenobiotics. Isolated mammalian liver cells have been extensively used in toxicological research. Fish hepatocytes have been isolated from many fish species (for review see Baksi and Frazier, 1990). A good yield of highly viable cells can easily be obtained and isolated fish hepatocytes are rather well characterized physiologically and biochemically (for review see Moon et al., 1985, Baksi and Frazier, 1990). They have been shown to have the capacity of oxidative metabolism by the cytochrome P-450 monooxygenase system (phase I reactions) as well as for conjugations (Andersson and Koivusaari, 1986, Buhler and Williams, 1988, Baksi and Frazier, 1990). Most of the studies carried out so far, have focused on xenobiotic metabolism and a minor part of the studies have been concerned with the use of freshly isolated fish hepatocytes in toxicity testings (for review see Baksi and Frazier, 1990).

The present study shows that the MEIC reference chemicals are considerably more toxic to *Daphnia magna* than to freshly isolated rainbow trout hepatocytes. The difference in toxicity exceeds one order of magnitude for most of the chemicals. A comparison of our toxicity data on *Daphnia*, on the other hand, with toxicity data on fish found in the literature revealed a surprisingly good agreement between *Daphnia* EC_{50} and fish LC_{50} values. The 3 h incubation time used in the hepatocyte test is short compared to the 24 h exposure used in the *Daphnia* test. Unfortunately the incubation time with freshly isolated rainbow trout hepatocytes can not be extended past 4–5 h without a significant reduction of the viability of the cells. As was indicated, however, in the comparison of our hepatocyte data with data from toxicity tests carried out with the MEIC reference chemicals on mammalian primary hepatocytes cultures (about 24 h incubation) the difference in sensitivity can not be attributed solely to the shorter incubation time. Even though a longer incubation time would be to prefer, because 3 h may possibly be too short for some types of cellular damages to manifest themselves, the comparison indicates that not much would be gained if a longer incubation time could be used. One fact that should be taken into consideration in trying to explain the lower sensitivity of the hepatocytes, is the fat content of the rainbow trout liver. The liver of hatchery rainbow trout may contain a relatively high amount of fat and it seems possible that highly apolar compounds can partition to a considerable amount into the fat depots in the hepatocytes. A part of the molecules taken up by the cell could be localized in the fat depots and thus be withdrawn from a possible interaction with targets in the cell. If this were the case, there should be a difference in the distribution of the values in relation to the regression line or the 1:1 correlation line (Fig. 1). The compounds with a high octanol/water partition coefficient should then tend to lie above the regression line or be distributed more far away from the line for 1:1 correlation. However, no such trend can bee seen in the distribution of the values. Thus a high fat content does not seem to significantly decrease the toxicity of the chemicals to the liver cells.

On the basis on the results obtained in our study, we can not for the present advocate the use of isolated rainbow trout hepatocytes in routine toxicity testing as a substitute for acute toxicity tests on fish. The endpoint, rubidium leakage, used in the present study should in the first place reflect an abolishment of the integrity of the plasma membrane or an increased membrane leakage due to 'cell death'. It is possible that this endpoint is too crude. We feel that parameters or combinations of parameters reflecting more subtle alterations in cell function should be explored before it can be decided whether or not isolated fish liver cells are useful in routine toxicity testing in aquatic toxicology. Preliminary studies in our laboratory indicate that parameters such as alteration in intracellular Ca^{2+} and glutathione concentration, reveal alterations in cellular functions at much lower concentrations of toxicants than rubidium leakage. Further experiments to find informative parameters which could be used in toxicity testing with fish liver cells are in progress.

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AQUATIC TOXICOLOGY

Comparison of P-4501A1 monooxygenase induction in gizzard shad (*Dorosoma cepedianum*) following intraperitoneal injection or continuous waterborne-exposure with benzo[a]pyrene: Temporal and dose-dependent studies

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Abstract

The objective of this study was to compare the dose-response and time-course for hepatic P-4501A1 induction in gizzard shad (*Dorosoma cepedianum*) following intraperitoneal (i.p.) injection or waterborne-exposure to benzo[a]pyrene (B[a]P). P-4501A1 activity was measured as ethoxyresorufin-*O*-deethylase (EROD) activity. To investigate the i.p. dose-response, fish were injected with a range of B[a]P concentrations (0.1, 1.0, 10 and 50 mg/kg) and sacrificed 72 h post-injection. In comparison, waterborne dose-response fish were exposed to a range of B[a]P concentrations (0.14, 0.24, 0.44 and 0.76 μg/L) and sacrificed after 10 d. The amount of B[a]P removed from the water by fish was estimated with a one-compartment linear clearance model. Maximum EROD activity was reached after fish were either injected with or cleared approximately 10 mg/kg. The injection doses (10 and 50 mg/kg) and waterborne concentrations (0.44 and 0.76 μg/L) that caused EROD induction were used to evaluate the time-course for EROD induction. Maximum EROD activity for both injection doses was reached by day 3 and for both waterborne concentrations by day 10. Although i.p. injections are adequate for rapid screening of potential P-4501A1 inducers over a wide dose range, continuous waterborne-exposures simulate a significant route of exposure and provide improved predictive capabilities for monitoring the time-course for P-4501A1 induction under environmentally realistic conditions.

Key words: Gizzard shad; EROD; Induction; Benzo[a]pyrene; Uptake

1. Introduction

Induction of cytochrome P-450 in fish can serve as a sensitive biological indicator for certain classes of environmental contaminants (Kleinow et al., 1987; Jimenez and

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