CENTRAAL ZIEKENHUISLABORATORIUM (CZL)

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REPORT NO: CZL/INDTOX/BBP 96-I/2

[redacted]

STUDY DETAILS

Sponsor:	[redacted]ica S.A./N.V.
Sponsor's representative:	Mark A. Martens
Study director:	Frank K. Martens
Date of issue:	14th December, 1996

STATEMENT OF CONFIDENTIALITY CLAIM

STATEMENT OF COMPLIANCE AND AUTHENTICATION

I, the undersigned, declare that the objectives formulated by the sponsor were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in the above study.
The study was not conducted according to the principles of Good Laboratory Practice as laid down by OECD (OECD Environment Monograph no 45, OECD/GD(92)32) but is in agreement with the best analytical practices. All laboratory notes pertaining to the development of this method are maintained in the archives of the laboratory and will be made available to the sponsor upon request.
The " Centraal Ziekenhuis Laboratorium" (CZL) has been accredited by the Belgian Ministery of Labour for industrial toxicology analyses (M.B. 10.09.1987 - S.B. 19.09.1987: Analysis of industrial raw materials, M.B. 23.03.1988 - S.B. 01.04.1988: Atmospheric monitoring, M.B. 30.11.1988 - S.B. 10.01.1989: Permormance of special biological investigations as referred to in the labour protection regulations) and is subjected to timely inspections and ring tests for clinical biochemistry as prescribed by Belgian law.

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Frank K. Martens, PhD, MD.
Study Director

4 th December, 1996

Date

STUDY CONTRIBUTIONS

Name	Title
Bart Lepla	Industrial engineer
Kristien Timperman	Medical technologist

CONTENTS

1. SUMMARY

A [redacted] sma for the study of the metabolism and pharmacokinetics of bu[redacted]experimental animals. 24 hour plasma samples were collected from immature (20-22 day old) female rats treated with 1, 10 and 100 mg/kg BBP via the oral and the subcutaneous route. This sighting study was conducted to allow the design of a main pharmacokinetics study in immature female rats. Immature female rats were used in the uterotrophic assay to determine the possible in-vivo oestrogenicity of BBP.
For the analysis acetonitrile was added to the plasma samples to precipitate the proteins and to mobilise BBP and its mono-esters. The mixture was centrifuged and the volume of the supernatant was reduced by evaporation. After addition of alkaline buffer, BBP was extracted with n-hexane. The organic layers were combined, dehydrated and evaporated to dryness. The residue was redissolved in methanol for GC-MS analysis.
The mono-esters which were maintained in solution in the alkaline phase were extracted with a mixture of n-hexane and dichloromethane after acidification.
The combined organic fractions were dehydrated and evaporated to dryness and the residue methylated by adding diazomethane dissolved in ether. After reaction the ether solution was evaporated and the residue redissolved in methanol for analysis by GC-MS.
The GC-MS apparatus was equipped with a 50 m apolar WCOT capillary column and full mass chromatograms were recorded from which specific ion chromatograms could be derived electronically. The m/z values selected for the analysis of BBP were 91 and 149, and the m/z values used for the analysis of the methylated mono-esters were 91, 149 and 163.
This study demonstrated that the method of analysis employed was not sufficiently sensitive to study the plasma kinetics of BBP and BeP beyond the 24 hour time point even at dose levels of 100 mg/kg. BuP levels could only be detected at the 100 mg/kg dose level. The findings of this preliminary study suggest that a dose level of 100 mg/kg should be considered for the study of the pharmacokinetics of BBP in immature female rats, if this method of analysis were to be used.

2. INTRODUCTION

Contradicting results have been obtained with BBP in a series of in-vitro and in-vivo oestrogenicity assays. The slightly positive results found in several in-vitro tests could not be reproduced in a well recognized in-vivo oestrogenicity model (i.e. uterotrophic assay) where BBP was administered via the oral and the subcutaneous route. In order to better understand this difference a pharmacokinetic programme was set up for immature female rats. This sighting study is the first step and should help in the design of a more complete study where the pharmacokinetics of BBP and its major metabolites will be studied in plasma and in urine. In this test (CTL project CO5695) 3 dose levels were considered for the oral and the subcutaneous route of administration each i.e. 1, 10 and 100 mg/kg. Three replicates were taken per dose group and

5 animals per replicate. The animals under study were 20-22 day old female rats of the Alderley Park Alpk APfSD Wistar strain, which is the same strain which was used for the uterotrophic assays. The animals were housed in metabolism cages for 24 hours after administration of the test substance. At 24 hours urine samples and blood samples were collected for chemical analysis. This report describes the analysis of BBP, BuP and BeP in the plasma samples.

3. EXPERIMENTAL

3.1 PLASMA SAMPLES

At 24 hours after oral and subcutaneous administration of BBP, blood was collected by means of 2.7 ml Monovette FE syringes with NaF and EDTA (Sarstedt). Plasma was separated by centrifugation immediately after collection of the blood samples and transferred into another Monovette tube. All plasma from rats of the same replicate were combined and allocated one code number. The combined plsama samples were immediately frozen and then shipped on dry ice to CZL for analysis.
All the plasma samples were received in good condition and were immediately stored at - 18 °C pending analysis. The code nos used are explained in the table below.

Route of administration	Dose (mg/kg)	Sample code
oral	1	1
		2
		3
	10	4
		5
		6
	100	7
		8
		9
subcutaneous	1	10
		11
		12
	10	13
		14
		15
	100	16
		17
		18

3.2 CHEMICAL ANALYSIS

The analysis of the plasma samples was conducted according to the method of analysis developed at CZL (1).

4. RESULTS

The concentrations of BBP, BuP and BeP in the plasma samples are shown in the table below.

Route of administration	Dose (mg/kg)	Sample code	BBP Conc. (mg/L)	BuP Conc. (mg/L)	BeP Conc. (mg/L)
oral	10	4	< LOD	< LOD	< LOD
		5	< LOD	< LOD	< LOD
		6	< LOD	< LOD	< LOD
	100	7	< LOD	??	< LOD
		8	< LOD	0.14	< LOD
		9	< LOD	0.13	< LOD
subcutaneous	10	13	< LOD	< LOD	< LOD
		14	< LOD	< LOD	< LOD
		15	< LOD	< LOD	< LOD
	100	16	< LOD	0.43	< LOD
		17	< LOD	0.28	< LOD
		18	< LOD	0.40	< LOD

For the method referred to in this report the LOD for BBP, BuP and BeP in plasma is 0.04 mg/L.

5. CONCLUSIONS

This preliminary study demonstrated that the current analytical method employed is not sufficiently sensitive for the study of the plasma kinetics of BBP and its mono-esters beyond the 24 hour time point . Only at the 100 mg/kg dose level BuP could be demonstrated in the sub mg/L range. These observations suggest that it would be necessary to use a dose level of at least 100 mg/kg to allow an adequate pharmacokinetic study of BBP and its mono-esters in the immature rat.

5. REFERENCES

(1) Report no CZL/INDTOX/BBP 96-I.