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Title:	In vitro investigations of the metabolic profile of Bemotrizinol by incubation of human hepatocytes and S9 fractions with 14C-Bemotrizinol
Project No.	Malibu (RD9559)
Compound No.	Bemotrizinol, 2,2´-[6-(4-Methoxyphenyl)-1,3,5-[ring-U-14C] triazine-2,4-diyl]bis[5-[(2-ethylhexyl)oxy]phenol]

Summary

The metabolic profile of bemotrizinol (BEMT) was investigated by incubation of 14C-BEMT with hepatocyte suspensions and liver S9 fractions of human origin. Aliquots of the incubation mixtures were removed after several time points, and proteins were precipitated by freezing (-80°C). Almost no radioactivity was recovered in the supernatants. Dioxane extraction of the precipitate resulted in a high extraction efficiency. The entire radioactivity was detected in the extracts as analyzed by liquid scintillation counting (LSC), and recovery in the extracts was found to be 100 ± 10 % throughout the experiments. Therefore, only the respective dioxane extract of the protein precipitate fractions was subjected to analysis by radio-HPLC.
Based on published literature and this laboratory's experience with liver in vitro metabolism systems, ^{14}C-BEMT was applied in target concentrations of 1 to 10 µM. A semiquantitative limit of detection (LOD) was determined for the radio-chromatograms and calculated to be 0.075 µM when using 10 µM ^{14}C-BEMT indicating that all peaks ≥ 1% are detected by the analytical method used.

This report consists of 35 pages

Distribution

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Lipoic acid (LA), 7-ethoxycoumarin, 7-hydroxycoumarin and 1-chloro-2,4-dinitrobenzene (CDNB) were used as positive controls in human hepatocytes and S9 preparations. With human hepatocytes, LA was metabolized extensively. In human liver S9 fractions supplemented with cofactors for phase I metabolic reactions, 7-ethoxycoumarin was metabolized, and a peak identified as 7-hydoxycoumarin was generated. With human S9 preparations (supplemented with co-factors for phase II), 7-hydroxycoumarin was converted to the corresponding conjugates, 7-hydroxycoumarinsulfate and 7-hydroxycoumarin glucuronide. CDNB was directly conjugated to the respective GSH conjugate of CDNB, when added to human S9 in the presence of glutathione (GSH). As a negative control (no hepatic metabolism), in all experiments heat deactivated control (HDC) samples were incubated with ^{14}C-BEMT in parallel with the "active" incubates (AI). The results demonstrate the validity of the applied in vitro systems and the chosen incubation conditions.

Incubations of ^{14}C-BEMT with human hepatocytes were done in concentrations of 1 and 10 µM. Chromatograms of samples collected directly after adding the test substance to the cell suspension (t=0h) clearly show one single peak of ^{14}C-BEMT for both BEMT concentrations, indicating the high purity of the test item and appropriate work-up of the samples as no immediate degradation products of the parent compound were generated. Also, comparing the 10 µM and 1 µM incubations, the tenfold lower concentration was nicely apparent from the tenfold lower signal in the chromatogram. After 4 hrs of incubation the chromatograms still showed only one single peak with comparable signal (peak area) of ^{14}C-BEMT for both concentrations as well as for the HDC sample, indicating the lack of any metabolic conversion or chemical degradation of BEMT in human liver cells. The absence of any additional radiopeak in the HDC sample demonstrates the stability of BEMT in the test system (no chemical degradation), while the result in the AI indicates the absence of any enzymatic metabolism of BEMT in human liver cells. Therefore, biotransformation of BEMT via phase I and/or phase II metabolic pathways was not observed in vitro using human hepatocytes.

To exclude that this result was solely based on limited uptake of BEMT into intact cells, additional in vitro experiments using human liver S9 preparations were performed. The S9 fraction is a mixture of unfractionated microsomes and cytosol containing a wide variety of drug-metabolizing enzymes and is commonly used as a preferred test system in several in vitro studies investigating phase I and phase II metabolism of a drug compound.

Human liver S9 preparations including the NADPH regenerating system were used to study the activity of cytochrome P450 enzymes (CYP), which catalyze the majority of phase I drug metabolism reactions. Incubations were performed using these S9 preparations with 10 µM ^{14}C-BEMT for 30 min, 2 h and 4 h incubation time. HDCs were run in parallel. Independent of incubation time the chromatograms showed only the single peak of ^{14}C-BEMT. This shows that BEMT does not undergo any phase I metabolism in this system.

Sulfate conjugation by phase II sulfotransferases (SULTs) in S9 fractions can be obtained in the presence of cofactor 3'-phosphoadenosine-5'-phosphosulphate (PAPS). Incubations using this system were performed with 10 µM ^{14}C-BEMT for 30 min, 2 h and 4 h. Beside the single peak of ^{14}C-BEMT in the chromatograms before incubation, after 30 min, 2 h and 4 h, no additional radiopeak was observed, which indicates the lack of sulfate-conjugation of ^{14}C-BEMT in the chosen system.

S9 fractions supplemented with cofactor Uridine 5'-Diphospho-Glucuronic acid (UDPGA) enable the conjugation reaction by UDP-glucuronosyltransferases (UGTs). Incubations using these preparations were performed with 10 µM ^{14}C-BEMT for 30 min, 2 h and 4 h. The radio-chromatograms showed just a single peak of ^{14}C-BEMT before incubation, after 30 min, 2 h and 4 h, which indicates the absence of any glucuronidation of BEMT.

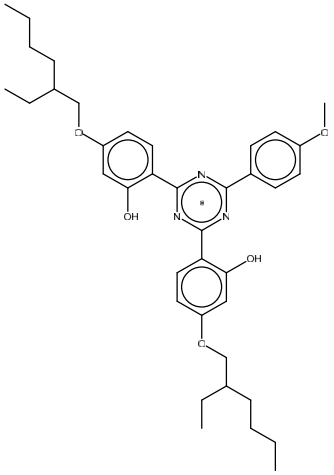
Incubations of 10 µM ^{14}C-BEMT with S9 preparations supplemented with glutathione (GSH) for 30 min, 2 h and 4 h revealed the absence of GSH conjugates. No direct reaction of ^{14}C-BEMT with GSH was observed.

To summarize, incubations of ^{14}C-BEMT with human hepatocyte and different human liver S9 preparations did not result in any phase I or phase II metabolites of BEMT in these well-established in vitro models.

Test Item

^{14}C-Bemotrizinol

Structural Formula



*** denotes [ring-U-^{14}C] label position**

Chemical Name

2,2´-[6-(4-Methoxyphenyl)-1,3,5-[ring-U-14C] triazine-2,4-diyl]bis[5-[(2-ethylhexyl)oxy]phenol]

Other Names /Abbreviations

^{14}C-BEMT
[triazine-U-^{14}C]BEMT

Chemical Purity/identity: 99.2%

Radiochemical Purity: 98.9%

Keywords
^{14}C-Bemotrizinol, Bemotrizinol, [triazine-U-^{14}C]BEMT, hepatocytes, S9, metabolism, in vitro, human

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Appendices

General Information

TITLE	*In vitro* investigations of the metabolic profile of Bemotrizinol by incubation of human hepatocytes and S9 fractions with ^{14}C-Bemotrizinol.
REPORT	No.: RD-00068522
STUDY DIRECTOR	Dr. Markus Fehr
MAIN AUTHOR	Dr. Markus Fehr
STUDY MONITOR	Dr. Mareike Beck
INVESTIGATORS	
Experimental and Analytical Chemistry	Albine Bompard
TEST FACILITY	DSM BD&T / Toxicology and Kinetics, ADME Labs Wurmisweg 576 CH-4303 Kaiseraugst, Switzerland
TEST SYSTEMS	*Cryopreserved pooled human hepatocytes*

Pool	100
Sex	50 Male, 50 Female
Supplier	XenoTech, Kansas City, KS, USA
Catalog No.	HCP100.H15
Lot No.	1910044
Data Sheet	01 May 2019
Cell Content	Min. 5.0 x 10^6 cells per vial
Viability	82%
Storage	In liquid nitrogen, vapor phase

Cryopreserved pooled human liver S9

Pool	Ultra Pool150
Sex	75 Male, 75 Female
Supplier	Corning Cell Culture Technology GmbH, S
Product Code	452116
Lot No.	38289
Data Sheet	03.2009
Protein Content	20 mg/ml
Storage	-80°C

TEST ITEM	[triazine-U-14C]BEMT
Synonym	^{14}C-Bemotrizinol
Supplier	Eurofins Selcia Limited, Ongar, Essex, UK
Lot No.	SEL/12312/2 Batch ID 12312KXM001-1

ANALYTICAL METHODS	Liquid scintillation counting (LSC), High Performance Liquid Chromatography (HPLC) with radiodetection
SAMPLES	Samples from incubations with cryopreserved hepatocyte suspensions and S9 liver fractions
STUDY DATES	
Experimental Start	11 Nov 2021
Experimental Termination	25 Jan 2022
STATEMENT OF AUTHENTICITY	The work and results reported in this document were gathered and prepared according to current scientific best practices and standards. The Study Director's (Main Author's) signature attests the veracity of the report and compliance to these standards.

1. Summary

The metabolic profile of bemotrizinol (BEMT) was investigated by incubation of ^{14}C-BEMT with hepatocyte suspensions and liver S9 fractions of human origin. Aliquots of the incubation mixtures were removed after several time points, and proteins were precipitated by freezing (-80°C). Almost no radioactivity was recovered in the supernatants. Dioxane extraction of the precipitate resulted in a high extraction efficiency. The entire radioactivity was detected in the extracts as analyzed by liquid scintillation counting (LSC), and recovery in the extracts was found to be 100 ± 10 % throughout the experiments. Therefore, only the respective dioxane extract of the protein precipitate fractions was subjected to analysis by radio-HPLC.

Based on published literature and this laboratory's experience with liver *in vitro* metabolism systems, ^{14}C-BEMT was applied in target concentrations of 1 to 10 µM. A semiquantitative limit of detection (LOD) was determined for the radio-chromatograms and calculated to be 0.075 µM when using 10 µM ^{14}C-BEMT indicating that all peaks ≥ 1% are detected by the analytical method used.

Lipoic acid (LA), 7-ethoxycoumarin, 7-hydroxycoumarin and 1-chloro-2,4-dinitrobenzene (CDNB) were used as positive controls in human hepatocytes and S9 preparations. With human hepatocytes, LA was metabolized extensively. In human liver S9 fractions supplemented with cofactors for phase I metabolic reactions, 7-ethoxycoumarin was metabolized, and a peak identified as 7-hydoxycoumarin was generated. With human S9 preparations (supplemented with co-factors for phase II), 7-hydroxycoumarin was converted to the corresponding conjugates, 7-hydroxycoumarinsulfate and 7-hydroxycoumarin glucuronide. CDNB was directly conjugated to the respective GSH conjugate of CDNB, when added to human S9 in the presence of glutathione (GSH). As a negative control (no hepatic metabolism), in all experiments heat deactivated control (HDC) samples were incubated with ^{14}C-BEMT in parallel with the "active" incubates (AI). The results demonstrate the validity of the applied *in vitro* systems and the chosen incubation conditions.

Incubations of ^{14}C-BEMT with human hepatocytes were done in concentrations of 1 and 10 µM. Chromatograms of samples collected directly after adding the test substance to the cell suspension (t=0h) clearly show one single peak of ^{14}C-BEMT for both BEMT concentrations, indicating the high purity of the test item and appropriate work-up of the samples as no immediate degradation products of the parent compound were generated. Also, comparing the 10 µM and 1 µM incubations, the tenfold lower concentration was nicely apparent from the tenfold lower signal in the chromatogram. After 4 hrs of incubation the chromatograms still showed only one single peak with comparable signal (peak area) of ^{14}C-BEMT for both concentrations as well as for the HDC sample, indicating the lack of any metabolic conversion or chemical degradation of BEMT in human liver cells. The absence of any additional radiopeak in the HDC sample demonstrates the stability of BEMT in the test system (no chemical degradation), while the result in the AI indicates the absence of any enzymatic metabolism of BEMT in human liver cells. Therefore, biotransformation of BEMT via phase I and/or phase II metabolic pathways was not observed *in vitro* using human hepatocytes.

To exclude that this result was solely based on limited uptake of BEMT into intact cells, additional *in vitro* experiments using human liver S9 preparations were performed. The S9 fraction is a

mixture of unfractionated microsomes and cytosol containing a wide variety of drug-metabolizing enzymes and is commonly used as a preferred test system in several *in vitro* studies investigating phase I and phase II metabolism of a drug compound.

Human liver S9 preparations including the NADPH regenerating system were used to study the activity of cytochrome P450 enzymes (CYP), which catalyze the majority of phase I drug metabolism reactions. Incubations were performed using these S9 preparations with 10 µM ^{14}C-BEMT for 30 min, 2 h and 4 h incubation time. HDCs were run in parallel. Independent of incubation time the chromatograms showed only the single peak of ^{14}C-BEMT. This shows that BEMT does not undergo any phase I metabolism in this system.

Sulfate conjugation by phase II sulfotransferases (SULTs) in S9 fractions can be obtained in the presence of cofactor 3'-phosphoadenosine-5'-phosphosulphate (PAPS). Incubations using this system were performed with 10 µM ^{14}C-BEMT for 30 min, 2 h and 4 h. Beside the single peak of ^{14}C-BEMT in the chromatograms before incubation, after 30 min, 2 h and 4 h, no additional radiopeak was observed, which indicates the lack of sulfate-conjugation of ^{14}C-BEMT in the chosen system.

S9 fractions supplemented with cofactor Uridine 5'-Diphospho-Glucuronic acid (UDPGA) enable the conjugation reaction by UDP-glucuronosyltransferases (UGTs). Incubations using these preparations were performed with 10 µM ^{14}C-BEMT for 30 min, 2 h and 4 h. The radio-chromatograms showed just a single peak of ^{14}C-BEMT before incubation, after 30 min, 2 h and 4 h, which indicates the absence of any glucuronidation of BEMT.

Incubations of 10 µM ^{14}C-BEMT with S9 preparations supplemented with glutathione (GSH) for 30 min, 2 h and 4 h revealed the absence of GSH conjugates. No direct reaction of ^{14}C-BEMT with GSH was observed.

To summarize, incubations of ^{14}C-BEMT with human hepatocyte and different human liver S9 preparations did not result in any phase I or phase II metabolites of BEMT in these well-established *in vitro* models.

2. Introduction

2.1 Background

Studies on the metabolism and kinetics of bemotrizinol (BEMT), an approved UV-filter in Europe, should provide relevant information if potential metabolites of BEMT have to be assessed separately. These are disproportionate metabolites, which are identified only in humans or present at higher plasma concentrations in humans than in any of the animal species used during standard nonclinical toxicology testing, identified in human plasma that should be considered for safety assessment. Human metabolites that can raise a safety concern are those present at greater than 10 percent of total drug-related exposure at steady state (FDA, 2020).

2.2 Objective

The objective of this study was to investigate *in vitro* the metabolic fate of ^{14}C-BEMT. For this purpose, *in vitro* incubations with primary human hepatocytes and liver S9 fractions of human origin were performed.

2.3 Selection of the doses/concentrations

Based on published literature and this laboratory's experience with liver *in vitro* metabolism systems, ^{14}C-BEMT was applied in target concentrations of 1 to 10 µM.

2.4 Test guidelines

This study is performed for investigative purposes with the aim to identify the metabolic profile of BEMT in humans as laid out in FDA´s Safety Testing of Drug Metabolites Guidance for Industry document (FDA, 2020).

2.5 Time schedule

Experimental Start	11. Nov 2021
Experimental Completion	25. Jan 2022
Study completion (Date of signature of the study report)	31. Mar 2022

2.6 Experimental Design

The metabolic profile of BEMT was investigated by incubation of ^{14}C-BEMT with hepatocyte suspensions and liver S9 fractions of human origin. Aliquots of the incubation mixtures were removed after different time points, the proteins were precipitated by freezing (-80°C) and precipitate extracts were analyzed by High Performance Liquid Chromatography (HPLC) with radiodetection. The distribution of radioactivity in the supernatant, the precipitate extract, and the protein pellet after treatment with tissue solubilizer was determined by liquid scintillation counting (LSC).

2.7 Abbreviations

ACN	Acetonitrile
AI	Active Incubate
BEMT	Bemotrizinol
CDNB	1-chloro-2,4-dinitrobenzene
CYP	Cytochrome P450 enzymes
g	Gravity
GSH	Glutathione
GST	Glutathione-S-transferase
HDC	Heat deactivated control
HPLC	High Performance Liquid Chromatography
LA (or ALA)	lipoic acid (α-lipoic acid)
LSC	Liquid scintillation counting
N	Background noise
PAPS	3'-phosphoadenosine-5'-phosphosulphate
PBS	Phosphate Buffered Saline
ROI	Region of interest
RT	Retention time on HPLC
S	Signal
SULT	Sulfotransferase
UDP	Uridine 5'-diphosphosphate
UGT	Uridine 5'-diphospho-glucuronosyltransferases

3. Materials and Methods

3.1 Test Systems

3.1.1 Human Hepatocytes

Cryopreserved pooled human hepatocytes

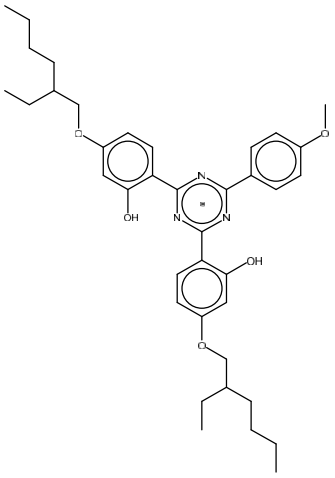
Pool	100
Sex	50 Male, 50 Female
Supplier	XenoTech, Kansas City, KS, USA
Catalog No.	HCP100.H15
Lot No.	1910044
Data Sheet	01.May.2019 (See Appendix 1)
Cell Content	Min. 5.0 x 10^6 cells per vial
Viability	82%
Storage	In liquid nitrogen, vapor phase

3.1.2 Human liver S9

Pool	Ultra Pool 150
Sex	75 Male, 75 Female
Supplier	Corning Cell Culture Technology GmbH, Schwerin, Germany
Product Code	452116
Lot No.	38289
Data Sheet	03.2009 (See Appendix 2)
Protein Content	20 mg/ml
Storage	-80°C

3.2 Test Item

Name ^{14}C-Bemotrizinol



*** denotes [ring-U-^{14}C] label position**

Chemical name	2,2′-[6-(4-Methoxyphenyl)-1,3,5-[ring-U-^{14}C] triazine-2,4-diyl]bis[5-[(2-ethylhexyl)oxy]phenol]
Synonym	^{14}C-BEMT
Supplier	Eurofins Selcia Limited, Ongar, Essex, UK
Lot No.	SEL/12312/2 Batch ID 12312KXM001-1
Molecular Weight	627.8 g/mol
logP	12.9 (predicted value, SciFinder)
Specific activity	46.96 mCi/mmol (1737 MBq/mmol) 74.6 µCi/mg (2.76 MBq/mg)
Date of manufacture	06 May 2021
Radiochemical purity	98.9% (HPLC)
Chemical purity	99.2% (HPLC at 217 nm)
Physical form	viscous yellow oil
Certificate of Analysis	See Appendix 3
Storage conditions	Glass bottle, < -20°C

3.3 Reference substance

Name	**^{14}C-Lipoic acid**
Chemical name	[dithiolanyl-5-^{14}C]-5-(1,2)dithiolan-3-yl-pentanoic acid
Molecular Weight	207.32
Batch/Lot-No.	11835EKC008-4
Supplier	Selcia Limited, Ongar, Essex, UK
Specific activity	31.35 mCi/mmol (1160 MBq/mmol) 151.2 µCi/mg (5.6 MBq/mg)
Date of manufacture	14 Oct 2020
Radiochemical purity	98.0% (HPLC)
Chemical purity	96.2% (HPLC at 217 nm)
Physical form	colorless solution in acetonitrile 0.925 mCi/mL (34.24 MBq/mL)
Storage conditions	Glass bottle, < -70°C

Name	**7-ethoxycoumarin**
Molecular Weight	190.2 g/mol
Batch/Lot-No.	60302/1
Supplier	Fluka; cat. no. 02644
Chemical purity	> 99 %
Physical form	powder
Storage conditions	Room temperature

Name	**7-hydroxycoumarin**
Molecular Weight	162.14 g/mol
Batch/Lot-No.	BCBW2757
Supplier	Sigma-Aldrich; cat. numb. 54826
Chemical purity	> 99 %
Physical form	Powder
Storage conditions	4°C

Name	**1-chloro-2,4-dinitrobenzene**
Molecular Weight	202.5 g/mol
Batch/Lot-No.	095M4077V
Supplier	Sigma-Aldrich; Cat. numb. CS0410
Storage conditions	-20°C

3.4 Test substance preparation

3.4.1 ^{14}C-BEMT

^{14}C-BEMT was delivered pure (viscous yellow oil). For the experiments dilutions were prepared in 1,4-dioxane resulting in stocks with concentrations around 50 mM of ^{14}C-BEMT.
^{14}C-BEMT-stock:
1.6 mg of the ^{14}C-BEMT (stored at -20°C) were dissolved with 1,4-dioxane resulting in a solution with a concentration of 52 mM, and 5.5 10^6 dpm/µL. ^{14}C-BEMT-stock was prepared Nov 11, 2021.
For the *in vitro* incubations, the target concentrations of 1 and 10 µM were prepared from diluted stock solutions.
For incubations with 10 µM test item,^{14}C-BEMT-stock was diluted 1:10 with 1,4-dioxane and then 1:5 with water to result in a concentration of 1 mM in 20% 1,4-dioxane. For the incubation ^{14}C-BEMT was diluted 1:100 in the respective incubation media (final solvent concentration of 0.2%).
For incubations with 1 µM test item, ^{14}C-BEMT-stock was diluted 1:50 with 1,4-dioxane and then 1:10 with water to result in a concentration of 0.1 mM in 10% 1,4-dioxane. For the incubation ^{14}C-BEMT was diluted 1:100 in the respective incubation media (final solvent concentration of 0.1%).

3.4.2 ^{14}C-Lipoic acid (positive control)

A solution of radiolabeled Lipoic acid (^{14}C-LA) was prepared in acetonitrile (8.1 mM) and further diluted 1:4 in water. For the incubation ^{14}C-LA was diluted 1:100 in the respective incubation media (final solvent concentration of 0.25%).

3.4.3 7-ethoxycoumarin (positive control for phase I metabolism)

A stock solution of 7-ethoxycoumarin was prepared at 50 mM in 95% ethanol and further diluted with water to a 1 mM solution in 4% ethanol. For the incubations 7-ethoxycoumarin was diluted 1:100 in the respective incubation media (final solvent concentrations of 0.2 to 0.04%).

3.4.4 7-hydroxycoumarin (positive control for phase II metabolism)

A stock solution of 7-hydroxycoumarin was prepared at 50 mM in 95% Ethanol and further diluted with water to a 1 mM solution in 2% Ethanol. For the incubation 7-hydroxycoumarin was diluted 1:100 in the respective incubation media (final solvent concentration of 0.02%).

3.4.5 1-chloro-2,4-dinitrobenzene (positive control for glutathione conjugation)

A 100 mM solution of 1-chloro-2,4-dinitrobenzene (CDNB) was used from the Sigma-Aldrich kit (CS0410).

3.5 Experimental Procedures

3.5.1 Hepatocytes suspensions

Media and thawing

Human pooled hepatocytes (Supplier SEKISUI-XenoTech; HCP100.H15 - lot 1910044)

Media

- OptiThaw Human Hepatocyte Kit: (XenoTech, Cat no. K8500)
- OptiIncubate Hepatocyte Media (XenoTech, Cat no. K8400)

Thawing:

- The vial containing hepatocytes taken from the liquid nitrogen tank was placed into a water bath at 37°C for around 80 sec until the frozen pellet moved freely in the tube.
- The pellet was transferred to 20-30 mL 37°C prewarmed OptiThaw medium in a Falcon tube; the tube was gently inverted until pellet was fully dispersed
- The tube was centrifuged at 100 x g for 5 min at room temperature. The supernatant was aspirated and discarded
- Cell pellet was parted/separated by gently tapping the tube and cells resuspended in 3 mL OptiIncubate medium.

Cell counting

To count the cells and to determine the cell viability, 50 µl of the cell suspension was added to an OptiCount vial containing 400 µl of PBS and 50 µl Trypan Blue (XenoTech, Kansas City, KS, USA).
After a few minutes' incubation at room temperature, 10 µl of the cell suspension was transferred to a counting chamber (4-Chip disposable Hemocytometer Neubauer improved. # LUN404. LuBio Science, Zürich, Switzerland), where viable and dead cells (stained in blue) were counted. The viability was determined as ratio between number of viable and number of total cells.

Hepatocyte incubations with ^{14}C-BEMT and sample workup procedure

Hepatocytes incubations were all performed in non-coated 12-well plates (Greiner bio-one; Cat no. 392-0047). ^{14}C-BEMT stock solutions were diluted with respective incubation medium to 2x of the target concentrations (1-10 µM), and 0.5 mL aliquots per well were distributed into the plate.

The cell suspensions were diluted to $2x10^6$ viable cells/mL with prewarmed medium, and incubation started by adding 0.5 mL aliquots of the cell suspension to the wells containing the test compound ^{14}C-BEMT. Final solvent (1,4-dioxane) concentrations in the incubations ranged from 0.1 to 0.2%, depending on the BEMT concentration. Plates with human hepatocytes were placed on an orbital shaker in an incubator, at 37°C, 5% CO_2, 95% humidity.
After defined incubation time points, aliquots were collected from the reaction vial and immediately frozen at -80°C to stop the reaction. Samples were kept at -80°C for at least 30 min and then centrifuged at 14000 rpm. The supernatant (aqueous phase) was collected, and its radioactivity content was determined. The precipitate (pellet) was extracted with the addition of an equal volume of 1,4-dioxane. The sample was vortexed and sonicated to improve solubilization and centrifuged at 14000 rpm. The 1,4-dioxane extract was finally collected for analysis with HPLC-radiodetection as well as for the measurement of radioactivity using LSC (determination of the radiobalance). The remaining pellet was dissolved with a tissue solubilizer (Soluene-350) for at least 4 hs at 40°C and analyzed with LSC to determine the amount of non-extractable radioactivity.

Hepatocyte incubations with ^{14}C-LA (positive controls)

For the experiment with human hepatocytes parallel incubations were performed using ^{14}C-LA as a positive control.
For each 1 mL hepatocyte incubation (10^6 cells/mL) 10 µL of a 2 mM solution of ^{14}C-LA (in 25% ACN) were added to 1 mL hepatocytes suspension (10^6 cells/mL) resulting in a final concentration of 20 µM ^{14}C-LA (in 0.25% ACN).
After defined time points aliquots were removed and immediately frozen at -80°C. After freezing for at least 30 minutes, and subsequent centrifugation (10 min, 14000 rpm), the supernatant was removed, and ^{14}C-LA was analyzed with a HPLC-radiodetector.

Heat deactivated control incubations (negative controls)

As a negative control (no hepatic metabolism), in all experiments heat deactivated human hepatocytes were incubated with ^{14}C-BEMT in parallel with the "active" incubations (AI).
For those heat deactivated controls (HDC), cells were boiled for 7 min at 80°C using a thermoblock. They were used in the incubation after cooling down.
Sample preparation and analysis was the same as for the active incubations.

3.5.2 Human S9 suspensions

Media and thawing

Human S9 (Supplier Corning Cell Culture Technology GmbH)

The frozen vial containing human S9 fraction was transferred directly from the Biofreezer (-80°C) into a water bath at 37°C. As soon as the fraction appeared homogeneous, the vial was put on ice until use.

Preparation of incubations mixtures for human S9 fractions

Incubation of test items with human S9 fractions were performed in 2 mL Eppendorf tubes.

- The incubation mixture for human S9 fraction to test CYP-enzyme activity consisted of:
  - 870 µL of incubation buffer (100 mM potassium phosphate buffer)
  - 50 µL solution A (NADPH regenerating system)
  - 10 µL of solution B (Glucose-6-Phosphate Dehydrogenase)
  - 10 µL of test substance
  - 10 µL of H_2O or 20% Dioxane in H_20
  - 50 µL of liver fraction (AI or HDC)

The incubation mixture for human S9 fraction to test SULT-enzyme activity consisted of:
  - 928 µL of incubation buffer (100 mM Tris HCl pH 7.5)
  - 27 µL of 9.4 mM PAPS solution
  - 10 µL of test substance
  - 10 µL of H_2O or 20% Dioxane in H_20
  - 25 µL of liver fraction (AI or HDC)

- The incubation mixture for human S9 fraction to test UGT-enzyme activity consisted of:
  - 650 µL of incubation buffer (100 mM potassium phosphate buffer)
  - 80 µL solution A (25 mM uridine 5'-diphospho-glucuronic acid)
  - 200 µL of solution B (250 mM Tris-HCL, 40 mM $MgCl_2$, 0.125 mg/mL alamethicin in H_2O)
  - 10 µL of test substance
  - 10 µL of H_2O or 20% Dioxane in H_2O
  - 50 µL of liver fraction (AI or HDC)

- The incubation mixture for human S9 fraction to test conjugation with GSH consisted of:
  - 920 µL of incubation buffer (100 mM potassium phosphate buffer)
  - 10 µL of 2 mM GSH solution (freshly prepared)
  - 10 µL of test substance
  - 10 µL of H_2O or 20% Dioxane in H_2O
  - 50 µL of liver fraction (AI or HDC)

The mixture was warmed in a water bath for 5 min at 37°C before the human S9 fraction was added, as the starting point of the reaction (t=0). Prepared samples were incubated for up to 4 hours and respective aliquots were collected at different time points.

Human S9 fractions incubations with ^{14}C-BEMT and sample workup procedure

After defined incubation time points, aliquots were collected from the reaction vial and immediately frozen at -80°C to stop the reaction. Samples were kept at -80°C for minimum 30 min and then centrifuged at 14000 rpm. The supernatant (aqueous phase) was collected, and its radioactivity content was determined. The precipitate (pellet) was extracted with the addition of an equal volume of 1,4 dioxane. The sample was vortexed and sonicated to improve solubilization and centrifuged at 14000 rpm. The dioxane extracted part was finally collected for analysis with HPLC-radiodetector as well as for the measurement of the radioactivity using LSC. The remaining pellet was dissolved with a tissue solubilizer (Soluene-350) for at least 4h at 40°C and analyzed with LSC to determine the amount of non-extractable radioactivity.

Human S9 fractions incubations with positive controls and sample workup procedure

After different incubation times, aliquots were collected and immediately frozen at -80°C. Samples were kept at -80°C for 30 min and then centrifuged at 14000 rpm. The supernatant (aqueous phase) was collected for analysis with HPLC-radiodetector or HPLC-diode-array detector (DAD).

3.6 Analytical Methods

3.6.1 Liquid Scintillation Counting

Aliquots of liquid samples were added to 10 mL Ultima Gold scintillation cocktail and counted directly in a Tri-carb 3100 TR (Canberra Packard, USA) liquid scintillation counter using the Transformed Spectral Index of the External Standard Spectrum (tSIE) method for quench correction.
Solid samples (e.g., protein precipitates) were dissolved in 1 mL Soluene 350 at 40°C for at least 4 h. After addition of 15 mL Ultima Gold the radioactivity was determined by LSC.

3.6.2 HPLC Analysis for ^{14}C-BEMT

HPLC system: Agilent 1260 Infinity II, Agilent
Detectors: Berthold Radioflow Detector LB 514 with YG 150 solid scintillation cell

Column: Lichrospher 100 RP-18 (5um) LichroCART 250-4
Merck KGaA, Darmstadt, Germany
Guard Column: Lichrospher 100 RP-18 (5um) LichroCART 4-4

Merck KGaA, Darmstadt, Germany
Flow: 1 mL/min
Gradient: Solvent A: 0.06 % ammonium formate, pH 4.6 (adjusted with formic acid)
Solvent B: 1,4-dioxane
Temperature: 35°C

time (min)	% A	% B
0	20	80
3	20	80
30	0	100
34	0	100
36	20	80
41	20	80

Peak integration was performed with the Berthold Radiostar 5.0.12.6 software. Peaks in the radio-chromatogram were integrated after background subtraction. The sum of integrated peaks (ROI = region of interest) was set to 100%.
Typical retention time for BEMT was around 20 min.

3.6.3 HPLC Analysis for ^{14}C-LA

HPLC system: Agilent 1100 Series,
Detectors: Agilent 1100 Series G1315B Diode Array Detector, at 325 nm
Berthold Radioflow Detector LB 509 with YG 150 solid scintillation cell

Column: Superspher 60 RP Select B - LiChroCART 250-4 (250 x 4 mm, 5 µm)
Merck KGaA, Darmstadt, Germany
Guard Column: Superspher 60 RP- Select B LiChroCART 10-2
Merck KGaA, Darmstadt, Germany
Flow: 0.5 mL/min
UV Read out: 325 and 230 nm
Gradient: Solvent A: 20 mM KH_2PO_4. pH 2.7 (adjusted with phosphoric acid)
Solvent B: 90% Acetonitrile in water. pH 2.7 (adjusted with phosphoric acid)

time (min)	% A	% B
0	100	0
6	100	0
16	90	10
61	80	20
91	50	50
101	20	80
111	0	100
121	0	100

3.6.4 HPLC Analysis for 7-ethoxycoumarin, 7-hydroxycoumarin, CDNB

HPLC system: Agilent 1100 Series,
Detectors: Agilent 1100 Series G1315B Diode Array Detector, at 235 nm
Berthold Radioflow Detector LB 509 with YG 150 solid scintillation cell
Column: Superspher 60 RP Select B - LiChroCART 250-4 (250 x 4 mm, 5 µm)
Merck KGaA, Darmstadt, Germany
Guard Column: Superspher 60 RP- Select B LiChroCART 10-2
Merck KGaA, Darmstadt, Germany
Flow: 0.7 mL/min
UV Read out: 325 and 230 nm
Gradient: Solvent A: 10 mM ammonium formate, pH 3.5 (adjusted with formic acid)
Solvent B: 100% Acetonitrile

time (min)	% A	% B
0	100	0
5	100	0
25	0	100
30	0	100
31	100	0
37	100	0

Peak integration was performed with the Berthold Radiostar 5.0.12.6 software. Peaks in the radio-chromatogram were integrated after background subtraction. The sum of integrated peaks (ROI = region of interest) was set to 100%.

Typical retention times for 7-hydroxycoumarin, 7-ethoxycoumarin and CDNB were around 18, 22 and 22 min, respectively.
After metabolic conjugation, retention times for 7-hydroxycoumarin glucuronide, 7-hydroxycoumarin sulfate, and the major CDNB conjugate were around 14 min, 15 min and 16 min, respectively.

4. Results and Discussion

4.1 ^{14}C-BEMT: stock solutions and stability

A radio-chromatogram of the ^{14}C-BEMT stock solution used in the *in vitro* incubations is shown in figure 1. ^{14}C-BEMT eluted around 20 min (retention time) in the HPLC system. No impurities were observed indicating a high stability of ^{14}C-BEMT.

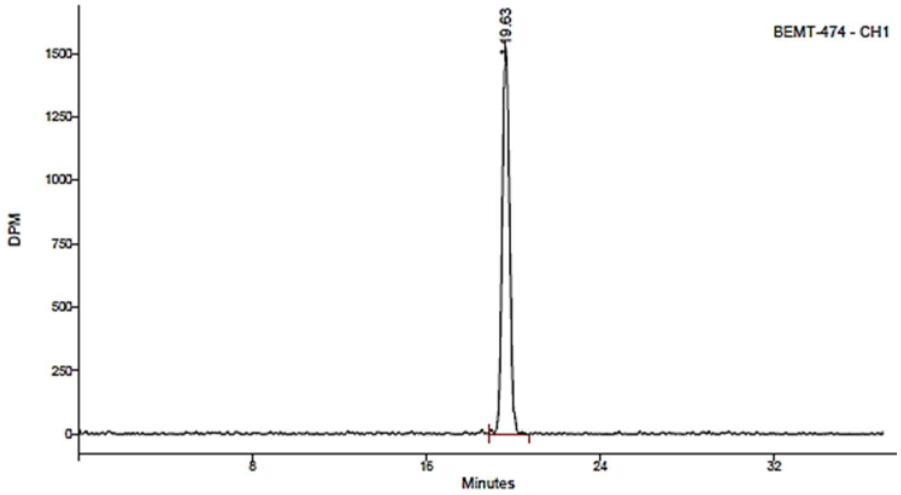


Figure 1: Radio-chromatogram after incubation of human hepatocytes with ^{14}C-BEMT

4.2 ^{14}C-BEMT: semiquantitative detection limit

A semiquantitative limit of detection (LOD) was determined to ensure that requirements of the FDA guideline were covered. The signal (S) and background noise (N) of 1 µM ^{14}C-BEMT was determined and calculated according to the common LOD formula: S / N = 3.
Measured values at 1 µM ^{14}C-BEMT concentration were S/N=200/5=40, resulting in an estimated LOD of 0.075 µM at S/N=3.
In other words, in incubations using 10 µM ^{14}C-BEMT with a semiquantitative LOD of 0.075 µM, all peaks ≥ 1% are detected by the analytical method, which fulfills the requirement of detecting metabolites ≥ 10%

4.3 *In vitro* hepatocyte incubations investigating ^{14}C-BEMT metabolism

For investigations of the *in vitro* metabolism of BEMT, ^{14}C-BEMT and ^{14}C-Lipoic acid were incubated in hepatocyte suspensions of human origin. The incubations were performed at 37°C with a viable cell concentration of 10^6 cells/mL. ^{14}C-BEMT was used at concentrations of 1 and 10 µM. Control incubations were performed in parallel with heat deactivated hepatocytes in each experiment to assess non-enzmatic reactions. Aliquot samples taken at different time points from the incubations were analyzed by radio-HPLC directly after incubation or after storage at -20°C.

A recovery/balance of radioactivity in samples was also determined to exclude the loss of radioactivity (e.g., by adsorption to surfaces or generation of volatile compounds).

4.3.1 Recovery of radioactivity and extraction efficiency

Radioactivity in samples taken at the beginning and at the end of the hepatocyte incubations was analyzed by liquid scintillation counting (LSC). For a recovery/balance during the sample work-up procedure, radioactivity in the supernatants after protein precipitation, and in the extract and residual pellet after dioxane extraction of the precipitate was determined by LSC. The extraction efficiency was good as the entire radioactivity was only detected in the dioxane extract. Recovery of radioactivity was acceptable throughout the experiments (100 ± 10 %) (data not shown).

4.3.2 ^{14}C-Lipoic acid (positive control)

Control experiments were performed using human hepatocytes with the positive control ^{14}C--Lipoic acid (^{14}C-LA) at a concentration of 20 µM. Radio-chromatograms of samples taken before and after 4 hrs of incubation are shown in figure 2.

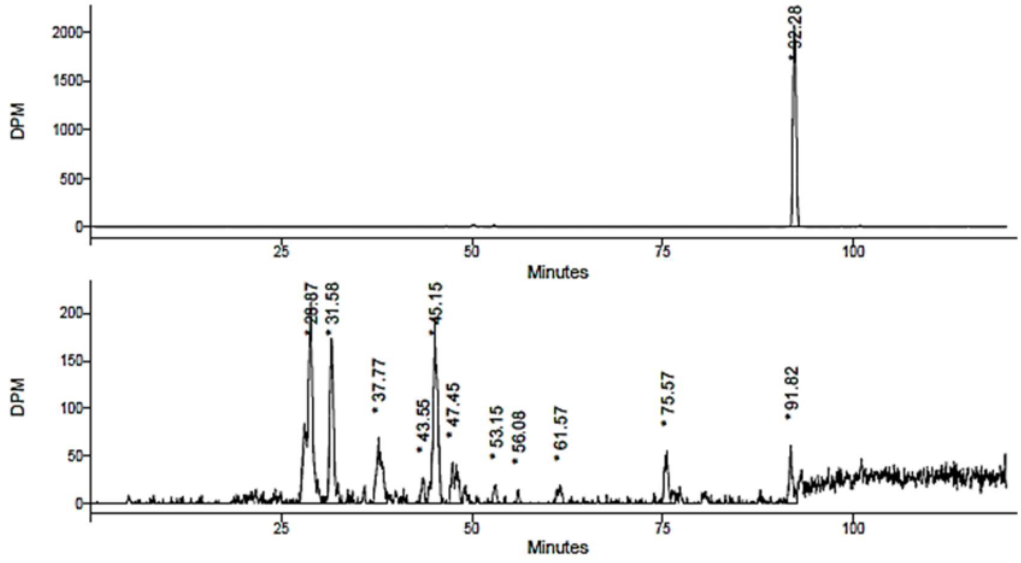


Figure 2. Radio-chromatograms after incubation of human hepatocytes with ^{14}C-lipoic acid before incubation (at the top) and after 4 h (at the bottom)

Before incubation ^{14}C-LA elutes in a single radio-peak at a retention time around 92-95 min. After 4 h incubation, the chromatogram shows about 10 additional peaks and a significant decrease of the parent compound. The generated metabolites are detected at retention times of about 26 until 75 min, indicating the generation of various polar metabolites as also reported in literature (Schupke et al. 2001). In conclusion, ^{14}C-LA was extensively metabolized with human hepatocytes under the chosen incubation conditions.

4.3.3 ^{14}C-BEMT metabolism using human hepatocytes

Incubations were performed using human hepatocytes with ^{14}C-BEMT concentrations of 1 and 10 µM (described in chapter 3.5.1). Radio-chromatograms of samples taken before and after 4 hrs of incubation of the active hepatocytes or HDCs are shown in the following figure 3.

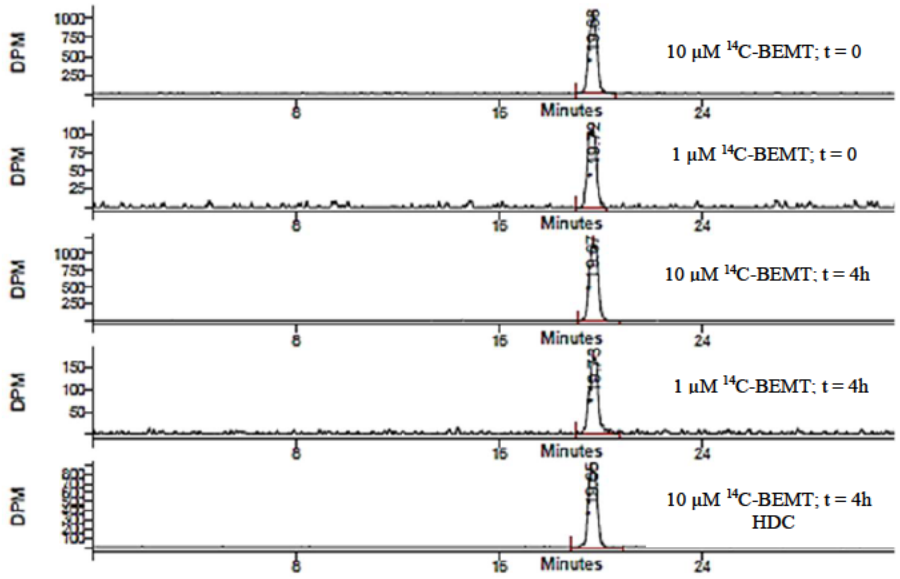


Figure 3: Radio-chromatograms before and after 4h incubation of human hepatocytes and HDC with ^{14}C-BEMT.

The chromatograms show the single peak of ^{14}C-BEMT before incubation (t = 0) for both BEMT concentrations. This indicates the high purity of the test item and appropriate work-up of the samples as no immediate degradation products of the parent compound were generated. Also, comparing the 10 µM and 1 µM incubations, the tenfold lower concentration is nicely apparent from the about tenfold lower signal in the chromatograms (24000 dpm and 2700 dpm, respectively). After 4 hrs of incubation the chromatograms still show only the single peak with a similar signal (peak area) of ^{14}C-BEMT for both concentrations as well as for the heat deactivated sample, indicating the lack of any metabolic conversion or chemical degradation of BEMT in human liver cells. The absence of any additional radiopeak in the heat deactivated sample demonstrates both the stability of BEMT in the test system (no chemical degradation) and the absence of any enzymatic metabolism of BEMT in the active human liver cells.
Therefore, biotransformation of BEMT via phase I and/or phase II metabolic pathways was not observed *in vitro* using human hepatocytes.

4.4 *In vitro* S9 incubations investigating ^{14}C-BEMT metabolism

Investigations of the *in vitro* metabolism of BEMT in hepatocyte suspensions of human origin suggested the absence of BEMT biotransformation via phase I and/or phase II metabolic pathways as reported above for the selected test system. To exclude that this result is solely based on limited uptake of BEMT into intact cells, additional *in vitro* experiments using liver S9 fractions were performed. The term S9 originates from the supernatant (S9) after centrifugation of liver homogenate at 9000 g (gravity) and it is well documented that S9 fractions offer a more complete representation of the metabolic profile compared to microsomes and cytosol, as they contain both Phase I and II enzymes (Fasinu et al. 2012)

4.4.1 Metabolism using S9 preparations with CYP-activity

Human S9 fractions including the NADPH regenerating system measure the activity of Cytochrome P450 enzymes (CYP), which are enzymes that are able to metabolize a large variety of xenobiotic substances. CYP enzymes are linked to a wide array of reactions including O-dealkylation, S-oxidation, epoxidation, and hydroxylation and are responsible for the majority of phase I drug metabolism reactions.

To demonstrate the activity of the S9 fractions including the NADPH regenerating system for phase 1 metabolic reactions, the positive control 7-ethoxycoumarin at a concentration of 50 µM was tested in parallel. Radio-chromatograms of samples taken before and after 4 hrs of incubation are shown in figure 4.

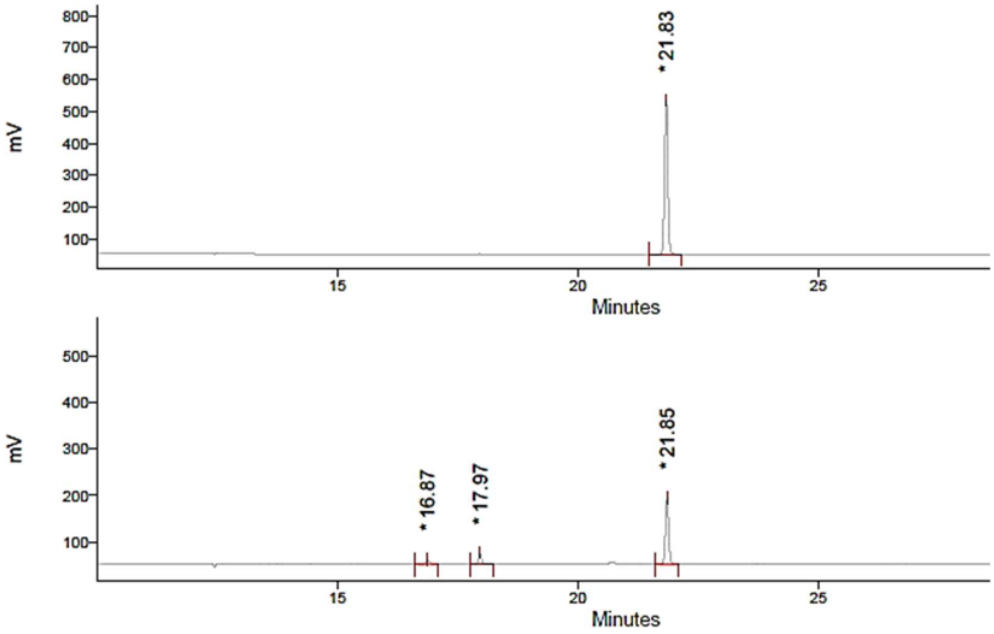


Figure 4: Chromatograms of human S9 fractions with 7-ethoxycoumarin before incubation (at the top) and after 4h of incubation (at the bottom).

The 7-ethoxycoumarin elutes at a retention time of about 22 min. After 4 h incubation with S9 fractions, this peak decreased significantly and 2 additional peaks at about 17 and 18 minutes were generated. The peak with the retention time of 17.97 min was identified as 7-hydroxycoumarin using a reference compound (not shown). The other metabolite eluting at about 17 min was not identified. The conversion of 7-ethoxycoumarin to 7-hydroxycoumarin is a known CYP-mediated reaction and shows the activity of the used S9 fraction.

Studies using human S9 fractions were performed with 10 µM ^{14}C-BEMT. Radio-chromatograms of samples taken after 30 min, 2 h and 4 h incubation times of the active incubates, or HDC samples after 4 h incubation, are shown in figure 5.

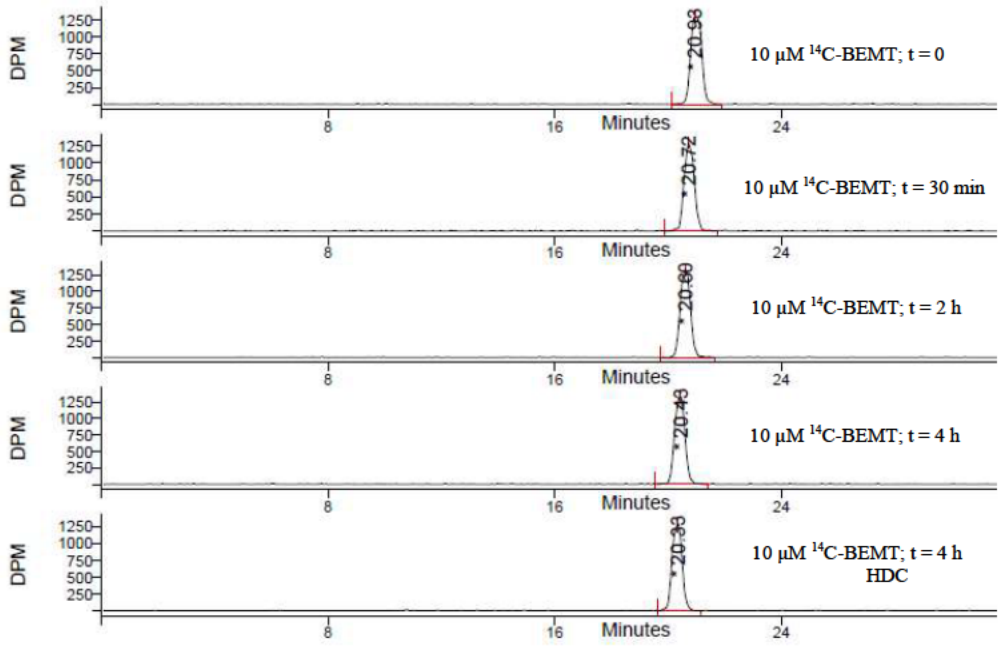


Figure 5: Radio-chromatograms after incubation of active human S9 fractions and HDC with ^{14}C-BEMT after different time points.

The chromatogram shows the single peak of ^{14}C-BEMT before incubation. After 30 min, 2 h and 4 h incubation with S9 fractions the chromatograms still show only the single peak of ^{14}C-BEMT, pointing to the lack of phase 1 metabolism reactions on BEMT in the S9 incubations. The heat deactivated control sample also resulted in the single ^{14}C-BEMT peak at about 20 min, showing that non-enzymatic reactions did not occur

Besides the absence of additional metabolite peaks no decrease in radioactivity of the parent compound ^{14}C-BEMT was observed (data not shown).

Therefore, biotransformation of BEMT via phase 1 metabolic pathways was not observed *in vitro* using S9 fractions including cofactors needed for CYPs' activity.

4.4.2 Metabolism using S9 preparations with SULT-activity

Human S9 fractions supplemented with cofactor 3'-phosphoadenosine-5'-phosphosulphate (PAPS) measure the activity of sulfotransferase (SULT) enzymes, which are a family of phase II enzymes transferring a sulfate group from PAPS to the hydroxyl group of an acceptor. Sulfonation is recognized as an important reaction in the metabolism of numerous xenobiotics, drugs, and endogenous compounds.

To demonstrate the activity of the chosen system of S9 fractions including the cofactor PAPS for phase II sulfonation reactions, the positive control 7-hydroxycoumarin at a concentration of 10 µM was tested in parallel. Radio-chromatograms of samples taken before and after 30 min of the active incubates or HDCs are shown in figure 6.

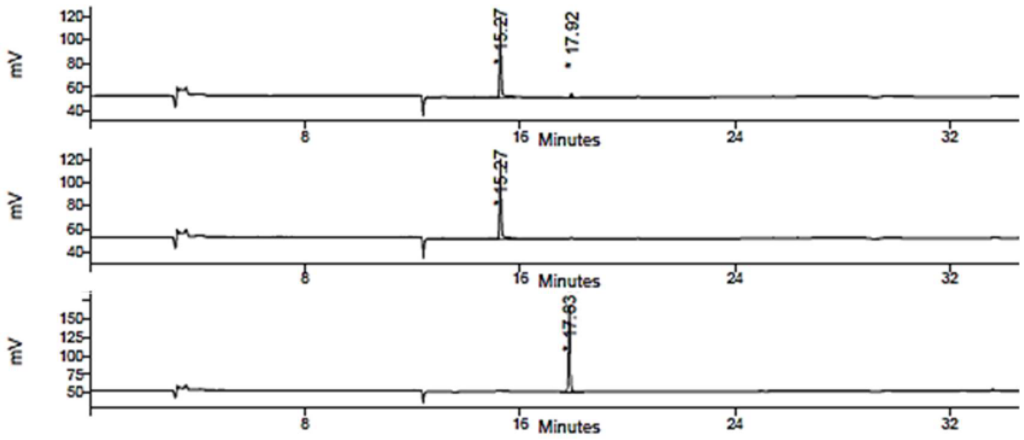


Figure 6: Chromatograms of human S9 fractions with 7-hydroxy coumarin before incubation (at the top), after 30 min (in the middle) and after 30 min of incubation with HDC (at the bottom).

The chromatograms of the AI samples show the immediate enzymatic reaction of of 7-hydroxycoumarin to the corresponding 7-hydroxycoumarin sulfate. 7-hydroxycoumarin with a retention time of 17.92 min was already significantly converted to the corresponding sulfate in the sample taken directly after initiating the incubation (t = 0) and sulfate conjugated completely after 30 min. The identity of 7-hydroxycoumarin sulfate with a retention time of 15.27 min was also confirmed with a reference substance (data not shown). In contrast, 7-hydroxycoumarin sulfate was not generated in the heat deactivated samples showing that the reaction was enzyme-mediated.

Incubations were performed using human liver S9 preparations (including PAPS) with 10 µM ^{14}C-BEMT. Radio-chromatograms of samples taken after 30 min, 2 h and 4 h incubation time of the active incubates, or HDC samples after 4 h incubation, are shown in figure 7.

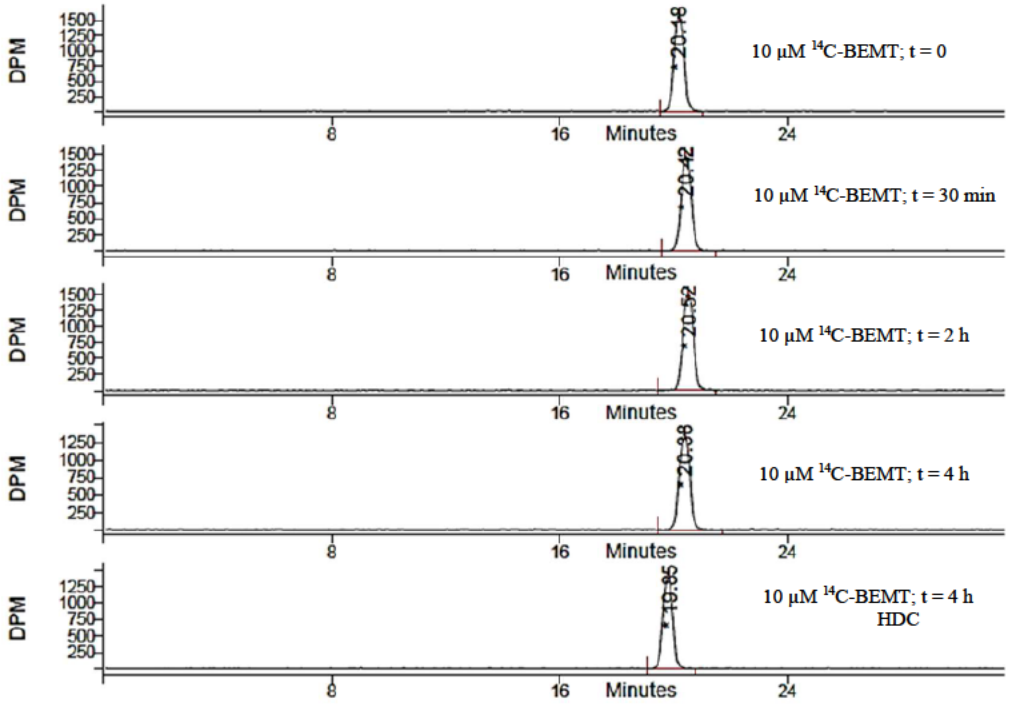


Figure 7: Radio-chromatograms after incubation of active human S9 fractions and HDC with ^{14}C-BEMT after different time points.

The chromatograms show the single peak of ^{14}C-BEMT before incubation, after 30 min, 2 h and 4 h. The absence of any metabolite peaks indicates the absence of sulfate conjugation reactions on BEMT. Also, no obvious decrease of the peak of ^{14}C-BEMT was observed.
The heat deactivated control sample also resulted in the single ^{14}C-BEMT peak at about 20 min, showing that non-enzymatic reactions did not occur.

Therefore, biotransformation of BEMT via phase II sulfate conjugation was not observed in this *in vitro* system.

4.4.3 Metabolism using S9 preparations with UGT-activity

Human liver S9 fractions supplemented with cofactor Uridine 5'-Diphospho-Glucuronic acid (UDPGA) mediate the activity of UDP-glucuronosyltransferase (UGTs) enzymes. These phase II enzymes catalyze the conjugation of glucuronic acid to xenobiotics with polar groups facilitating their clearance.

The activity of the S9 fractions including the cofactor UDPGA for phase II glucuronidation reactions was tested by using the positive control 7-hydroxycoumarin at a concentration of 10 µM. Radio-chromatograms of samples taken after 4 hrs of incubation of the active incubates or HDCs are shown in figure 8.

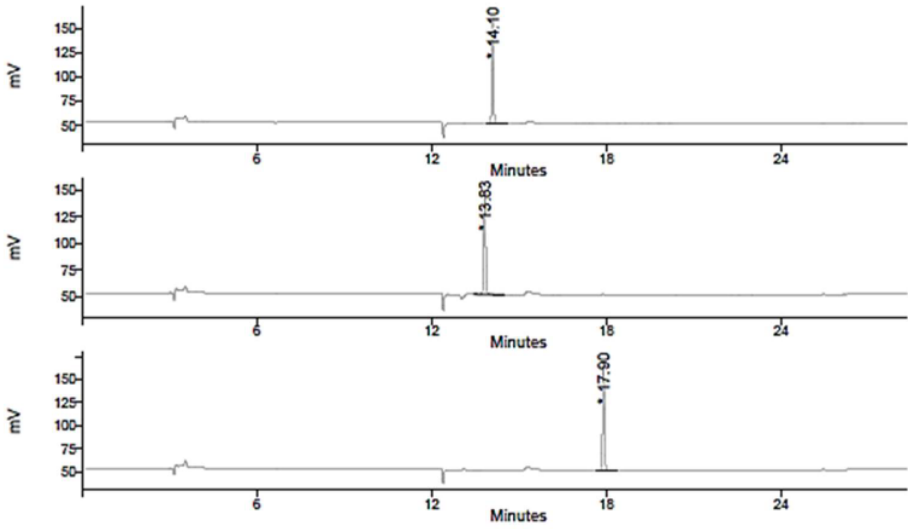


Figure 8: Chromatograms of human S9 fractions with 7-hydroxy coumarin before incubation (at the top), after 2 h (in the middle) and after 2 h of incubation with HDC (at the bottom).

The chromatograms clearly show the immediate enzymatic reaction of 7-hydroxycoumarin to the corresponding 7-hydroxycoumarin glucuronide. The 7-hydroxycoumarin with a retention time at 17.9 min was already completely converted to the corresponding glucuronide after less than 1 min (T0). 7-hydroxycoumarin glucuronide with a retention time of around 14 min was also identified with a reference substance (data not shown). In contrast, 7-hydroxycoumarin glucuronide was not generated in the heat deactivated samples showing that the reaction was enzyme-mediated.

Incubations were performed using human S9 preparations (including UDPGA) with 10 µM ^{14}C-BEMT. Radio-chromatograms of samples taken after 30 min, 2 h and 4 h incubation time of the active incubates, or HDC samples after 4 h incubation, are shown in figure 9.

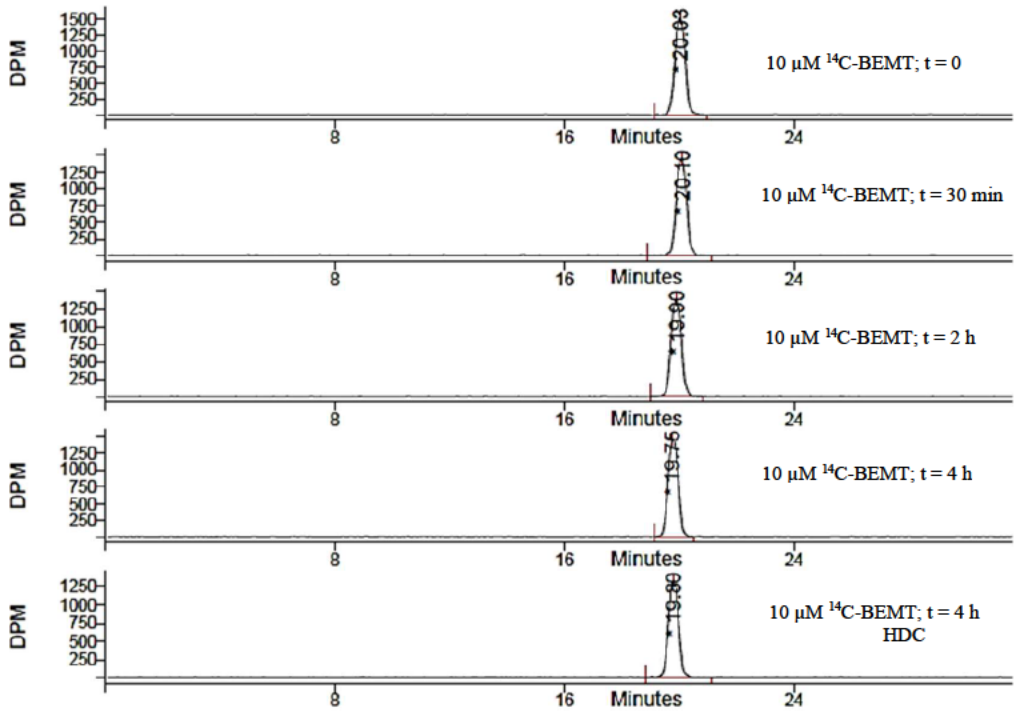


Figure 9: Radio-chromatograms after incubation of active human S9 fractions and HDC with ^{14}C-BEMT after different time points.

The chromatograms show the single peak of ^{14}C-BEMT before incubation, after 30 min, 2 h and 4 h, indicating the absence of glucuronidation reactions. The heat deactivated control sample also resulted in the single ^{14}C-BEMT peak at about 20 min, showing that non-enzymatic reactions did not occur.
Besides the absence of additional metabolite peaks no obvious decrease of the parent compound peak of ^{14}C-BEMT was observed.

Therefore, biotransformation of BEMT via phase II glucuronidation was not observed *in vitro* using cofactor supplemented S9 fractions.

4.4.1 Metabolism using S9 preparations with GSH

Glutathione (GSH) as an antioxidant facilitates metabolism of xenobiotics, either by direct reaction with reactive electrophiles or via Glutathione S-transferase (GST) enzymes that catalyze its conjugation to lipophilic xenobiotics.

The activity of the S9 fractions supplemented with and without GSH was shown in parallel incubations using the positive control CDNB at a concentration of 100 µM. CDNB is known to form GSH conjugates (Temellini et al., 1995) by nucleophilic aromatic substitution (Zheng & Ornstein, 1997).

Chromatograms of CDNB samples taken after 2 hrs incubation are shown figure 10.

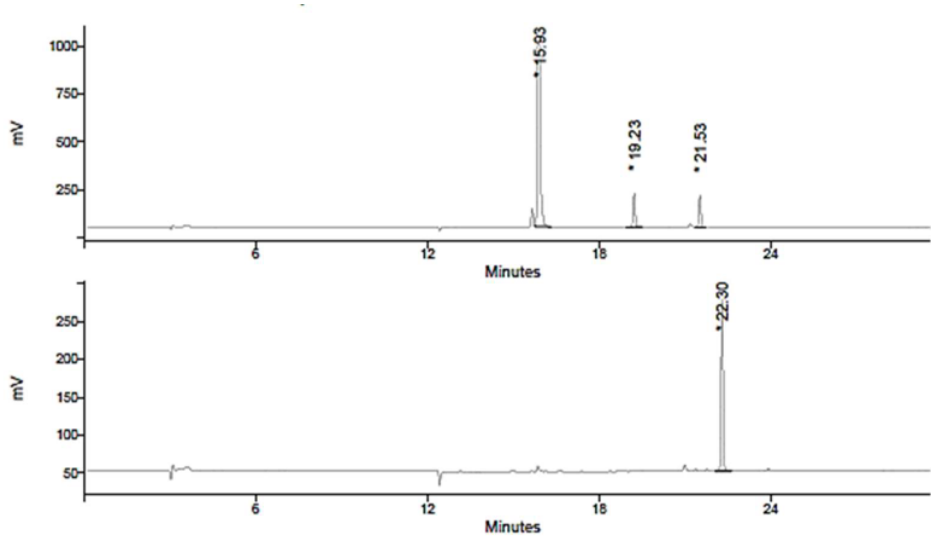


Figure 10: Chromatograms of human S9 fractions with CDNB and GSH after 2h (at the top) and without the addition of GSH (at the bottom).

The upper chromatogram shows the decrease of the CDNB peak in the presence of GSH and the appearance of two additional peaks were generated, a major peak with a retention time at about 16 min and a minor peak at about 19 min. In contrast, without the addition of GSH, the CDNB peak remained unchanged after 2 hrs.

Incubations were performed using human liver S9 fractions supplemented with GSH with 10 µM ^{14}C-BEMT. Radio-chromatograms of samples taken over 30 min, 2 h and 4 h incubation time of the active incubates, or HDC samples after 4 h incubation, are shown in figure 11.

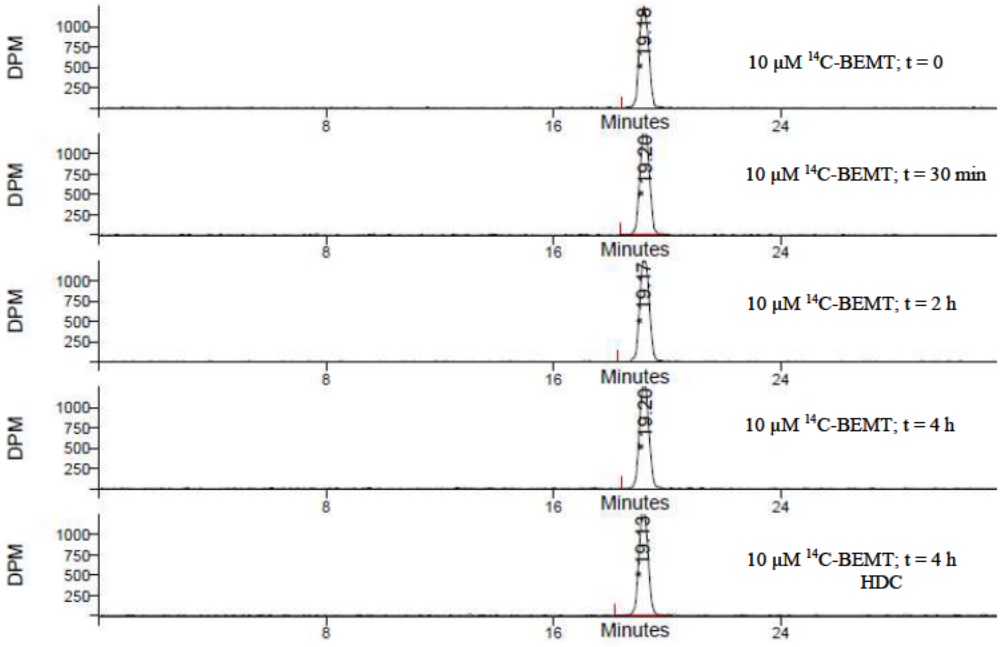


Figure 11: Radio-chromatograms after incubation of active human S9 fractions and HDC with ^{14}C-BEMT after different time points.

Again, the chromatograms show only the single peak of ^{14}C-BEMT before incubation, and after 30 min, 2 h and 4 hrs, clearly indicating the metabolic stability of BEMT and no formation of GSH conjugates.

5. Conclusion

Incubations of ^{14}C-BEMT with human hepatocytes and with different human liver S9 preparations did not result in any phase I and II biotransformation metabolites in these well-established *in vitro* models.

6. References

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Teichert J, Hermann R, Ruus P, Preiss R (2003) Plasma kinetics, metabolism, and urinary excretion of alpha-lipoic acid following oral administration in healthy volunteers. J Clin Pharmacol 43(11):1257-1267.

Temellini A, Castiglioni M, Giuliani L, Mussi A, Giulianotti PC, Pietrabissa A, Angeletti CA, Mosca F, Pacifici GM. Glutathione conjugation with 1-chloro-2,4-dinitrobenzene (CDNB): interindividual variability in human liver, lung, kidney and intestine. Int J Clin Pharmacol Ther. 1995 Sep;33(9):498-503.

Zheng, Y-J, Ornstein, R. L, Mechanism of Nucleophilic Aromatic Substitution of 1-Chloro-2,4-dinitrobenzene by Glutathione in the Gas Phase and in Solution. Implications for the Mode of Action of Glutathione S-Transferases. J. Am. Chem. Soc. 1997, 119, 4, 648–655.

7. Appendices

Appendix 1 Cryopreserved Human Hepatocytes Datasheet

SEKISUI XENOTECH

HEPATOSURE™

Data Sheet

HCP100.H15 Lot No. 1910044

Pool of 100 (50 Males and 50 Females)

Assured Minimum Yield: 5.0 x 10^6 cells per vial
Viability: 82%

This product was pooled from individual human hepatocytes that have been frozen and thawed. The yield and viability given above are based on experiments performed at XenoTech using our thawing protocol that includes a density gradient fractionation and the K8000 OptiThaw Kit. Details of XenoTech's hepatocyte thawing protocol can be found at www.xenotech.com. It is recommended to use XenoTech's thawing protocol, which includes a density gradient step, to maximize the viability of the recovered cells.

Enzyme	*Marker Substrate Reaction*	*[S] (µM)*	*Rate (pmol/million cells/min)*
CYP1A2	Phenacetin *O*-dealkylation	100	44.3 ± 2.5
CYP2A6	Coumarin 7-hydroxylation	50	56.6 ± 2.9
CYP2B6	Bupropion hydroxylation	500	29.2 ± 4.1
CYP2C8	Amodiaquine *N*-dealkylation	20	235 ± 57
CYP2C9	Diclofenac 4'-hydroxylation	100	217 ± 7
CYP2C19	*S*-Mephenytoin 4'-hydroxylation	400	9.86 ± 0.07
CYP2D6	Dextromethorphan *O*-demethylation	80	37.0 ± 4.8
CYP2E1	Chlorzoxazone 6-hydroxylation	500	67.2 ± 2.4
CYP3A4/5	Testosterone 6β-hydroxylation	250	167 ± 6
CYP3A4/5	Midazolam 1'-hydroxylation	30	36.7 ± 0.9
UGT	7-Hydroxycoumarin glucuronidation	100	239 ± 31
SULT	7-Hydroxycoumarin sulfonation	100	8.52 ± 0.67

Values for enzyme activities were determined at a single substrate concentration run with triplicate determinations.

To measure cytochrome P450 (CYP), UDP-glucuronosyl transferase (UGT) and sulfotransferase (SULT) activities, hepatocytes (1 x 10^6 cells/mL) in suspension were incubated in triplicate at 37 ± 1°C for 30 minutes in OptiIncubate and marker substrate, at the final concentrations indicated. Metabolite formation was determined by validated LC-MS/MS methods with deuterated metabolites as internal standards.

Uptake Activity Data

Uptake Transporter	*Marker Substrate*	*[S] (µM)*	*Rate (pmol/million cells/min)*
OATP1B1	Estrone sulfate	1	TBD
OATP1B3	CCK-8	1	TBD
OCT1	MPP+	1	TBD
NTCP	TCA	1	TBD

To measure uptake activities, hepatocytes (1.0 x 10^6 cells/mL) in suspension were incubated in triplicate at 4°C and 37°C for 1 minute in Krebs-Henseleit buffer and marker substrate, at the final concentrations indicated. Uptake of substrate was measured by scintillation counter.

Donor Information

Gender:	Males (50), Females (50)
Age:	8-74 years of age
Race:	Caucasian (80), African American (8), Asian (3), Hispanic (9)
Cause of Death:	Anoxia (33), Head trauma (25), Cerebrovascular accident (42)
Anitbody to Cytomegalovirus (CMV):	Positive (65), Negative (35)

All donors tested negative for Human Immunodeficiency Virus (HIV), Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus, and Rapid Plasma Reagin.

Store in liquid nitrogen, vapor phase

CAUTION: This sample should be considered as a potential biohazard and universal precautions should be followed. Intended for *in vitro* use only.

These data were generated by and are the property of XenoTech. These data are not to be reproduced, published or distributed without the express written consent of XenoTech.

Data sheet prepared 01 May 2019

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Appendix 2 Cryopreserved Human S9 Datasheet

Corning® UltraPool™ Human S9 150

Catalog No.	**452116**
Lot No.	38289
Qty./Package	**1.0 ml**
Protein Content	20 mg/mL in 150 mM KCL, 50 mM Tris (pH 7.5), 2 mM EDTA
Storage Conditions	**Store at -80 C**
Release Date	2009 March
Expiration Date	**2019 March**

Assay Results

Enzyme Measured	Assay	Enzyme Activity [in pmol/(mg x min)]
CYP1A2	Phenacetin O-deethylase	160
CYP2A6	Coumarin 7-hydroxylase	230
CYP2B6	(S)-Mephenytoin N-demethylase	14
CYP2C8	Paclitaxel 6α-hydroxylase	70
CYP2C9	Diclofenac 4'-hydroxylase	710
CYP2C19	(S)-Mephenytoin 4'-hydroxylase	22
CYP2D6	Bufuralol 1'-hydroxylase (the amount activity inhibited by 1µM quinidine)	24
CYP2E1	Chlorzoxazone 6-hydroxylase	840
CYP3A	Testosterone 6β-hydroxylase	1200

- All cytochrome P450 assays conducted at 1.6 mg/ml protein (except CYP3A4 which was at 1 mg/ml) with an NADPH generating system (1.3 mM NADP, 3.3 mM glucose 6-phosphate and 0.4 U/ml glucose 6-phosphate dehydrogenase), 3.3 mM $MgCl_2$, and incubated for 20 minutes or 10 minutes (CYP2C8, CYP2C9 and CYP3A4). 0.1 M Potassium phosphate buffer (pH 7.4) was used for all P450 enzymes except CYP2B6, CYP2C8, CYP2C19 (0.05 M) and CYP2A6, CYP2C9 which used 0.1 M Tris (pH 7.5).
- The pool is comprised of equal milligrams of S9 per donor.
- **HAZARD WARNING:** This S9 preparation was prepared from freshly frozen human tissues. All donor tissues have tested negative for pathogens by PCR for the following: HIV I/II, HTLV I/II, CMV, HBV and HCV, however, we recommend that this material be considered a potential biohazard.
- Donors with positive serology for CMV are identified in the donor demographic sheet with a single asterisk. Donors with CMV serology unknown are identified with a double asterisk. Donors CMV negative for serology are unmarked.

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Appendix 3 Certificate of Analysis for 14C-BEMT

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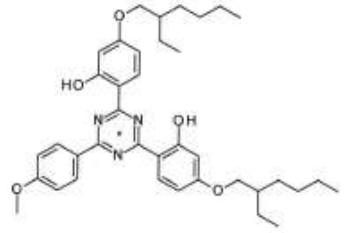
CERTIFICATE OF ANALYSIS

Analysis Reference	Document Revision	Page
SEL/12312/2	1	1 of 1



* denotes [ring-U-^{14}C] label position

Common name	[triazine-U-^{14}C]BEMT
Chemical name	2,2'-[6-(4-Methoxyphenyl)-1,3,5-[ring-U-^{14}C] triazine-2,4-diyl]bis[5-[(2-ethylhexyl)oxy]phenol]
Batch ID	12312KXM001-1
Molecular formula	$C_{36}H_{49}N_3O_5$
Storage conditions	Glass bottle, below -15 °C
Date of Manufacture	06 May 2021
Date of Analysis	07 May 2021

Analyses were performed in accordance with internal Quality Program procedures by the Analytical Support Group and the Radiochemistry Department of Eurofins Selcia Ltd. Comparison was with a reference sample (batch 17PS080015) where appropriate.

Test	Method	Result
Appearance & physical form	Visual inspection	Viscous yellow oil
Structure, identity & residual solvents	^{1}H NMR	Compatible with proposed structure & comparable with supplied reference Total detected residual solvents 0.7% w/w
Structure & identity	LC-MS	Compatible with proposed structure & comparable with supplied reference
Radiochemical purity	HPLC with radio detection	98.9%
Chemical purity & identity	HPLC with UV detection (330 nm)	99.2% Retention time comparable with supplied reference sample
Specific activity & labelled molecular weight	Gravimetric analysis by LSC	46.96 mCi/mmol 1737 MBq/mmol 74.6 µCi/mg 2.76 MBq/mg 629.32 g/mol @ 46.96 mCi/mmol

Issuer: Date: 18 May 2021

S. Yau, B.Sc.
Group Leader, Analytical Support

Reviewer: Date: 18 May 2021

S. J. Knight, Ph.D., MRQA
Quality Assurance

Caution: Radioactive material for research use only. Not suitable for human use.
Expiry date not determined. In the absence of stability data a purity check is recommended before use

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