An HPLC-UV Method Supporting the Reactivity Assessment of an IVA Acyl-Glucuronide Derivative

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ABSTRACT

Introduction: An important route of carboxylic acid metabolism is glucurono-conjugation yielding to the 1-O-β-acyl-glucuronide derivative. This is unstable at physiological and alkaline pH, undergoing with different rates 1.) intramolecular rearrangement leading to acyl-isomers, 2.) hydrolysis with release of the parent drug, 3.) adduct formation with tissues and plasma proteins leading to potential toxicity (i.e. hepatic toxicities, autoimmune responses). It was described that the covalent binding extent could be correlated with the 1-O-β-acyl-glucuronide global degradation rate: a short half-life (<1.5h) associated with a main acyl-migration is considered as a marker for more reactive acyl-glucuronides than those with long half-lives.

Objective: The 1-O-β-acyl-glucuronide degradation can only be demonstrated with a fine chromatography method ensuring the separation to its acyl-isomers and parent. This represents a challenge because of their very similar chemical structures. The aim of the study was to develop an HPLC-UV analytical method to assess the *in vitro* acyl-glucuronide degradation of the IVA compound (an Inventiva clinical drug candidate).

Method and Results: 1-O-β-acyl-glucuronide incubations (Phosphate buffer 0.1M pH7.4) were stopped at several time-points by a 2-fold dilution with acetonitrile / 1% HCOOH. 20µL were injected on a column XTerra MSC18, 150 x 3mm, 3.5µm. Separation of the 1-O-β-acyl-glucuronide, each of its three isomers and the parent was obtained with a gradient method from 80:20 to 10:90 (A: [Ammonium acetate 5mM / HCOOH 1%] – B: [Acetonitrile / HCOOH 1%]), for 18 minutes with a UV detection at 260nm, leading to retention times of 13.2, 11.4, 12.7, 13.6 and 16.1 minutes, respectively. Linearity was demonstrated from 0.6 to 60µM for the 1-O-β-acyl-glucuronide and its parent. A similar UV-response was assumed for each isomer due to the lack of available standards.

Method developments were also performed for acyl-glucuronide derivatives of known references (diclofenac, (S)-ibuprofen, indomethacin) used for ranking of the IVA 1-O-β-acyl-glucuronide reactivity.

OBJECTIVES

The 1-O-β-acyl-glucuronide degradation can only be demonstrated with a fine chromatography method ensuring the separation to its acyl-isomers and parent. This represents a challenge because of their very similar chemical structures. The aim of the study was to develop an HPLC-UV analytical method to assess the *in vitro* acyl-glucuronide degradation of an IVA compound (an Inventiva clinical drug candidate).

ABBREVIATIONS

°C	degree Celsius	H_2O	water	mL	milliliter
µL	micro liter	HCOOH	formic acid	mm	millimeter
µm	micrometer	HPLC	high-performance liquid chromatography	nm	nanometer
a	slope	KH_2PO_4	monopotassium phosphate	pH	
ACN	acetonitrile	KOH	potassium hydroxide	s	second
b	intercept	L	liter	ULOQ	upper limit of quantitation
DS	dilution solvent	LLOQ	lower limit of quantitation	UV	ultraviolet
g	gram	M	molar	v	volume
h	hour	min	minute		

METHOD

The IVA 1-O-β-acyl-glucuronide was incubated at 37°C, in KH_2PO_4 buffer. At each incubation time, samples were analyzed with an HPLC and UV detection method.

The IVA 1-O-β-acyl-glucuronide global degradation rate was calculated from the decreased IVA 1-O-β-acyl-glucuronide concentrations with time of incubation. IVA 1-O-β-acyl-glucuronide hydrolysis rate was calculated from IVA formed with time of incubation and IVA 1-O-β-acyl-glucuronide isomerisation rate was assessed by quantification of each IVA acyl-glucuronide isomer formed with time of incubation. 1-O-β-acyl-glucuronide incubations (Phosphate buffer 0.1 M pH7.4) were stopped at several time-points by a 2-fold dilution with acetonitrile / 1% HCOOH. 20µL were injected on a column XTerra MSC18, 150 x 3 mm, 3.5 µm. Separation of the 1-O-β-acyl-glucuronide, each of its three isomers and the parent was obtained with a gradient method from 80:20 to 10:90 (A: [Ammonium acetate 5 mM / HCOOH 1%] – B: [Acetonitrile / HCOOH 1%]), for 18 minutes with an UV detection at 260 nm.

Equipment:

Instrument	Type	Manufacturer
Autosampler	SIL-20AC	Shimadzu
HPLC-pump	LC20AD	Shimadzu
Column oven	CT0 20AC	Shimadzu
UV detector	SPD20A	Shimadzu
Analytical Column	XTerra MSC18, 150 x 3 mm, 3.5 µm	Waters

Chromatographic conditions:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow (mL/min)
0	80	20	
12	60	40	
14	10	90	0.5
18	10	90	
18.5	80	20	
21	80	20	
Acquisition time (min)		21	
Temperature (°C)	Oven: 45		Autosampler: 10
Injection volume (µl)		20	
UV wavelength (nm)		260	

Sample treatment:

Samples were diluted with acidified acetonitrile before analysis:

300 µL of ACN + 1% HCOOH were added directly into the tubes containing the sample. Samples were vortexed for 10 s and then were transferred into the vials for injection onto the chromatographic system.

Data evaluation:

The calibration curves were calculated from the peak area of analyte and the nominal analyte concentrations using a linear regression $y = a + bx$ with $1/x^2$ weighting .

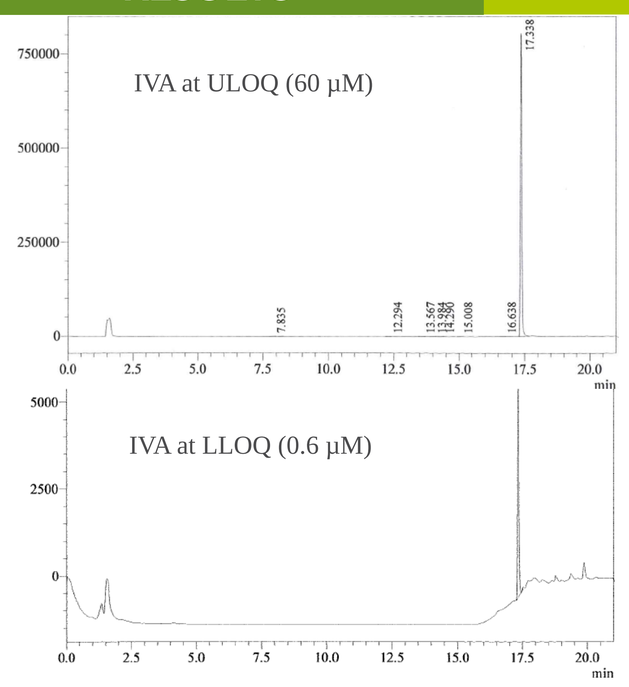
Preparation of solvents:

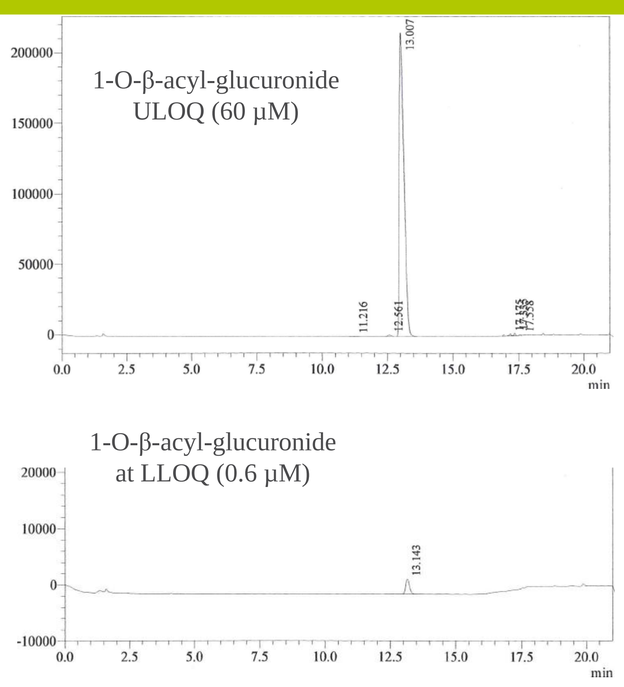
Solvent	Preparation
Ammonium acetate buffer 1 M	Weigh accurately 77.08 g of ammonium acetate into a 1000 mL volumetric flask. Bring to volume with water. (Store at room temperature for up to 1 year)
Solvent A	Ammonium acetate 5 mM / HCOOH (99.99:0.01; v/v) — Ammonium acetate buffer 1 M: 5 mL; HCOOH: 100 µL; H_2O: up to 1 L
Solvent B	Acetonitrile / HCOOH (99.99:0.01; v/v) — HCOOH: 100 µL; ACN: up to 1 L
Mobile Phase A	Solvent A / Solvent B (95:5; v/v)
Mobile Phase B	Solvent A / Solvent B (5:95; v/v)
Dilution solvent = DS	(Acetonitrile + 0.1% HCOOH) / (H_2O + 0.1% HCOOH) (50:50; v/v)
Rinsing solvent (autosampler)	Methanol / water (50:50; v/v)
Tris buffer pH 7.4	Weigh 15.76 g of Trizma hydrochloride into a 1000 ml volumetric flask. Add 800 ml of purified water and adjust pH at 7.4 with NaOH 2N. Then complete to 1000 ml with purified water
Phosphate buffer 0.1 M pH 7.4	Weigh 6.805 g of KH_2PO_4 into a 500 ml volumetric flask. Add 400 ml of purified water and adjust pH at 7.4 with KOH 1 M. Then complete to 500 ml with purified water.

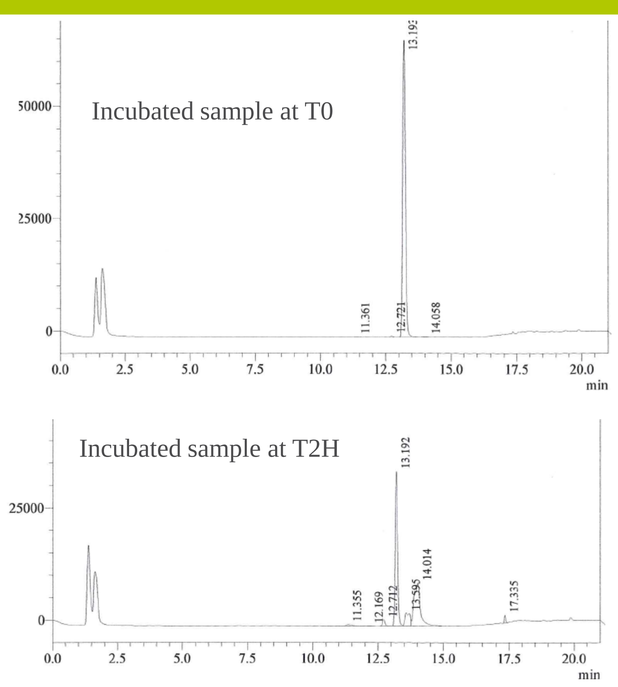
Calibration samples preparation:

Calibration Level	Solution Used	Volume Taken [µL]	Volume of DS Solvent Added [µL]	Final Concentration [µM]
ST5	SMG	300	700	60
ST4	ST5	300	300	30
ST3	ST5	100	900	6
ST2	ST4	100	900	3
ST1	ST3	100	900	0.6

RESULTS







DISCUSSION

An HPLC-UV analytical method was developped to assess the *in vitro* acyl-glucuronide degradation of the IVA compound (an Inventiva clinical drug candidate).

Separation of the 1-O-β-acyl-glucuronide, each of its three isomers and the parent was obtained, leading to retention times of 13.2, 11.4, 12.7, 13.6 and 16.1 minutes, respectively.

Linearity was demonstrated from 0.6 to 60 µM for the 1-O-β-acyl-glucuronide and its parent.

The *in vitro* intrinsic reactivity of the IVA acyl-glucuronide was therefore assessed by determining the global degradation rate in Phosphate buffer 0.1 M pH 7.4.

By comparison, the IVA acyl-glucuronide global degradation rate of ~2.4h was longer than the cut-off of 1.5 h proposed in the literature, and ranked between indomethacin acyl-glucuronide (1.7 h) and (S)-ibuprofen acyl-glucuronide (3.7 h) which are not known to be reactive *in vivo*.

Those observations lead to the conclusion that IVA acyl-glucuronide is not likely to react with proteins.

Kinetics of 1-O-β-acyl-glucuronide globaldegradation, hydrolysis and acyl migration: