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Standardization of a LC/MS/MS Method for the Determination of Acyl Glucuronides and Their Isomers

Acyl glucuronides of carboxylic acids are unstable and reactive metabolites that can isomerize (by acyl migration) and hydrolyze at physiological pH. They also can bind to proteins under these conditions. The reactivity of acyl glucuronides was determined in vitro *by monitoring the formation of isomers and hydrolysis products of 1-O-β-acyl glucuronide produced by human microsomes. This required the development of a standard analytical method for acyl glucuronides. Although ion spray-tandem mass spectrometry could be used for specific detection of acyl glucuronides, the isomers showed similar fragmentation pathways. Consequently, chromatographic separation was also required. The chromatographic method was based on a single stationary phase with variation of the percentage of the organic modifier in mobile phase (ammonium acetate 10mM - acetonitrile) in gradient elution mode. This method was used to resolve the acyl glucuronide isomers of eight drugs and to quantify the unmetabolized aglycones. The relative amounts of the different isomers were used to elucidate the mechanism of the isomerization reaction. This method can readily be extended to study the reactivity of the acyl glucuronide metabolites of new chemical entities.*

Introduction

Many acidic drugs containing carboxylic acid groups are metabolized to reactive acyl glucuronides. These metabolites are unstable at physiological pH and can both hydrolyse to form free aglycone and isomerize by acyl migration. Acyl migration involves transfer of the acyl group from the position 1 to the C-2, C-3 or C-4 position of the glucuronic acid ring, which results in formation of isomeric acyl glucuronides (1). These glucuronide isomers have been shown to bind covalently to proteins *in vitro* and *in vivo* causing potential toxicity (2). Therefore, models for predicting the toxicity of glucuronide derivatives of new chemical entities would be highly beneficial tools for drug discovery support programs.

The work discussed in this article was part of a project that involved developing a screening model for reactivity of acyl glucuronides based on production of acyl glucuronides by human microsomes, together with determining hydrolysis and isomerization rate constants and the extent of covalent binding with HSA (3). The model was evaluated using eight acidic drugs: Tolmetin, Zomepirac, Diclofenac, Fenoprofen, Ketoprofen, Ibuprofen, Suprofen and Furosemide (***F1***). These drugs have been extensively studied (4-11) and span a wide range of reactivity. Tolmetin and Zomepirac have been withdrawn from the market due to hypersensitivity reactions, whereas Ibuprofen is considered the safest NSAID and Furosemide shows only very low levels of covalent binding. The results presented here discuss developing a standard analytical method that allows the chromatographic separation and detection of the unmetabolized aglycone, the 1-O- acyl glucuronide metabolites and its isomers.

Materials and Method

In vitro *biosynthesis of acyl glucuronides*

The test compounds (Tolmetin, Zomepirac, Fenoprofen, Ketoprofen, Ibuprofen, Suprofen, Diclofenac and Furosemide) (400 µM) were incubated in triplicate for 4 hours at 37° C with human liver microsomes (3 mg/mL) in 100 mM tris buffer, pH 7.4, containing 1% DMSO, 5mM $MgCl_2$, 5 mM UDPGA (glucuronidation cofactor), and 1 mM UDPAG (reagent favoring glucuronidation). Aliquots were withdrawn at time points 0 (T_0) and 4 hr

(T_{4hr}). The reaction was stopped by protein precipitation with addition of trifluoroacetic (pH lowered to 3-4) and then centrifuged at 1500 rpm for 10 minutes.

LC/MS/MS analytical method

The incubation samples generated for each drug were analyzed by a generic LC/MS/MS method. The analytical column was a hypersil BDS (125 x 4 mm ID, Thermoquest). Separation of the different acyl glucuronides isomers from the aglycone was achieved using a gradient elution method. The mobile phase was a mixture of acetonitrile and 10 mM acetate ammonium buffer (70:30, v:v) + 0.5% acid acetic for solvent A and acetonitrile and 10 mM acetate ammonium buffer (4:96, v:v) for solvent B. The gradient profile was adjusted for each compound with a flow rate of 1 mL/min and run time was around 15 min. Detection and quantification were performed by tandem mass spectrometry using a turbo ion spray ion source (API 365, Applied Biosystems, Toronto, Canada).

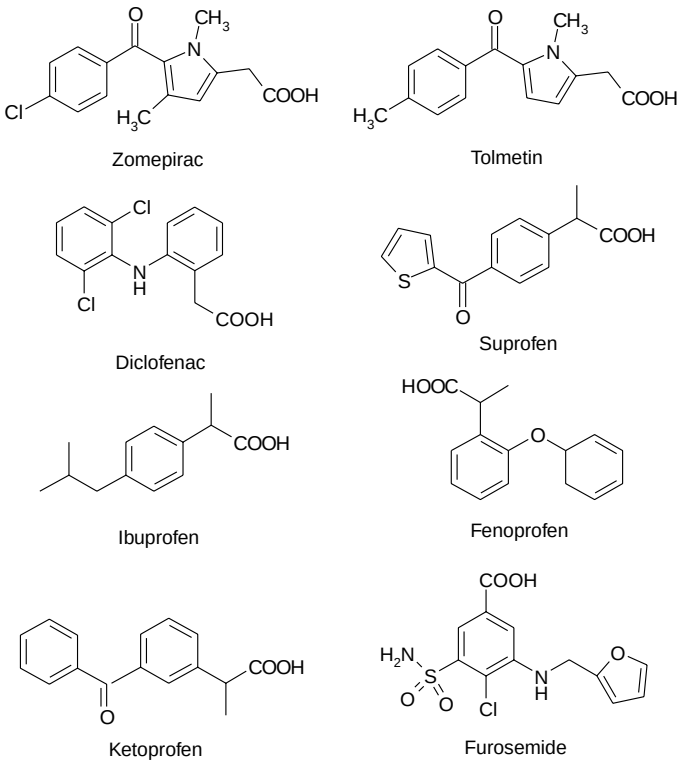
The percentage of aglycone biotransformation into acyl glucuronide was determined by quantification of the aglycone remaining after the four hour incubation period. The 1-O- -acyl glucuronide peak was identified by monitoring its disappearance after two hours of incubation with bovine - glucuronidase at 37° C. Finally, the percentage of the different acyl glucuronide isomers was assessed using peak area ratios.

Discussion

Tandem mass spectrometry conditions were optimized for each aglycone (***T1***). The proposed fragmentation pathways for the acyl glucuronide for the different ionization modes are outlined in ***F2*** (positive ionization mode) and ***F3*** (negative ionization mode). (Note that acyl glucuronides are not commercially

F1.

Molecular structures of the eight drugs studied.



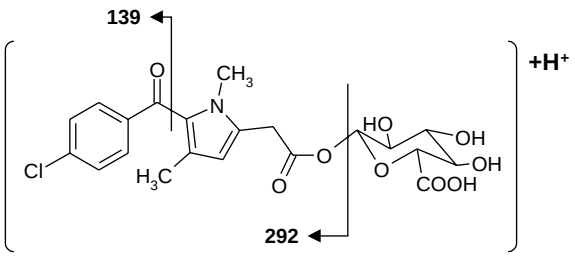
T1.

Mass spectrometry conditions for aglycone detection. [1]Ion Spray voltage (V); [2]Declustering Potential (V) or Orifice Voltage; [3]Focusing Potential (V) or Focusing Ring Potential; [4]Entrance Potential (V) or QO potential; E_{coll} = Collision Energy

	Mass Spectrometry Conditions					m/z	
Compound	IS[1]	DP[2]	FP[3]	EP[4]	E_{coll} (eV)	Q1	Q3
Tolmetin	+5700	11	240	-5.0	17	258	119
Zomepirac	+5800	17	170	-6.5	25	292	139
Diclofenac	+5900	27	270	-10.0	27	296	215
Suprofen	+5800	20	200	-8.0	29	261	111
Ketoprofen	+5900	66	290	-4.0	19	255	209
Fenoprofen	-4500	-6	-150	5.0	11	241	197
Ibuprofen	-4200	-66-	320	10.5	6.0	205	159
Furosemide	-4000	-16	-110	7.0	12	329	285

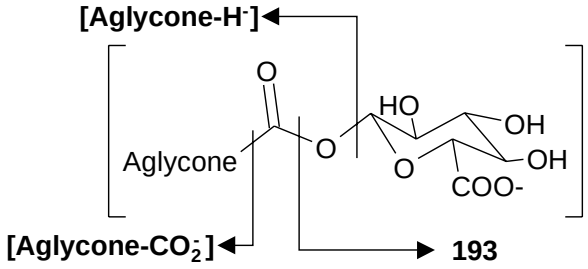
F2.

Proposed major fragmentation pathway for acyl glucuronides (e.g., Zomepirac) using TIS/MS/MS in positive ionization mode. Major fragments are the protonated aglycone ion (m/z = 292) and a fragment of the aglycone (m/z = 139).



available, and hence comparison samples cannot be run.) Liquid chromatographic conditions were standardized as much as possible, using the same column and same mobile phase composition. A gradient elution mode was needed for complete separation of acyl glucuronide isomers. Since the molecular structures of the tested compounds were very different, the gradient profile had to be optimized for each drug (***T2***). Some samples showed more than four peaks, which was attributed to the selectivity of the column for anomeric or enantiomeric forms. Therefore, identification of each acyl glucuronide isomer was not straightforward. Incubation of these samples with bovine -glucuronidase at 37 °C allowed identification of the 1-O-acyl glucuronide (***F4*** and ***F5***). The concentration of unmetabolized aglycone and the proportion of the acyl glucuronide isomers are shown in ***T3*** and ***T4***, respectively. Linearity was established for the different incubation media (i.e., microsomes and bovine -glucuronidase) over a concentration range of 0.05 - 10 µg/mL (0.005 - 1 µg/mL for Ibuprofen) with a correlation coefficient of 0.9976 or better for all the compounds studied.

F3.
Proposed major fragmentation pathway for acyl glucuronides using TIS/MS/MS in negative ionization mode. Major fragments include the deprotonated aglycone ion and the aglycone fragment generated by the loss of CO_2. The charge can also be located on the glucuronic acid moiety (m/z = 193).



T2.
LC gradient elution profile for tested drug.
Eluent A: ACN-acetate ammonium buffer 10 mM (70:30, v:v) +0,5% acetic acid
Eluent B: ACN-acetate ammonium buffer 10 mM (4:96, v:v)

Compound	Time	% Eluent A	% Eluent B
Tolmetin	0 - 6 min	90	10
	6 min	70	30
	13 min	70	30
	19 min	90	10
Zomepirac	0-13 min	100	0
Suprofen	0 - 7 min	93	7
	7 min	85	15
	13 min	85	85
	20 min	93	7
Diclofenac	0 - 13 min	100	0
Fenoprofen	0 - 32 min	100	0
Ibuprofen	0 - 5 min	100	0
	5 - 6 min	80	20
	6 - 11 min	80	20
	11 - 12 min	100	0
	12 - 17 min	100	0
Ketoprofen	0 - 15 min	91	9
Furosemide	0 - 10 min	70	30

Conclusion

The analytical method presented in this work will be used to set up a screening model for the reactivity of acyl glucuronides, as it can be used to determine the hydrolysis and isomerization constant rates for biosynthesised acyl glucuronides. These data will allow development of a reactivity scale for predicting protein binding potential of new chemical entities (3).

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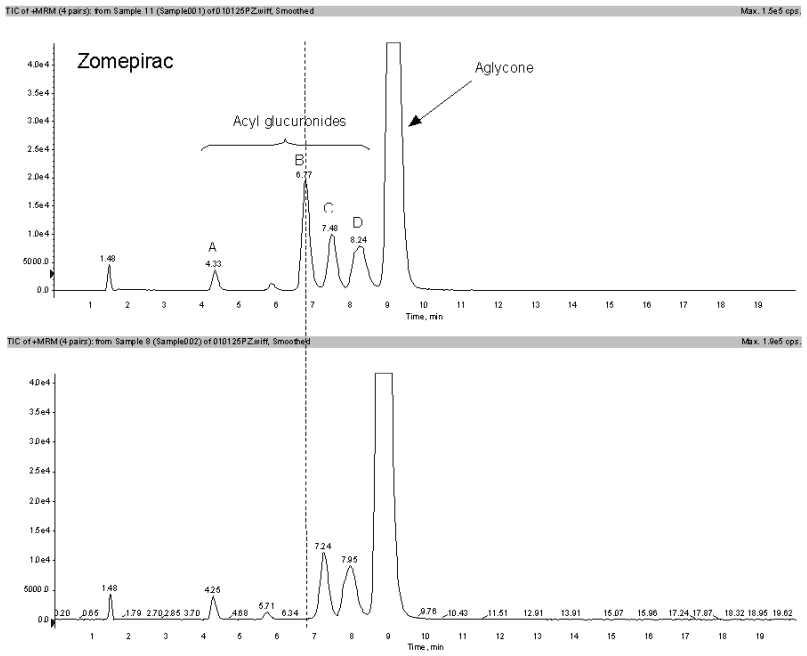
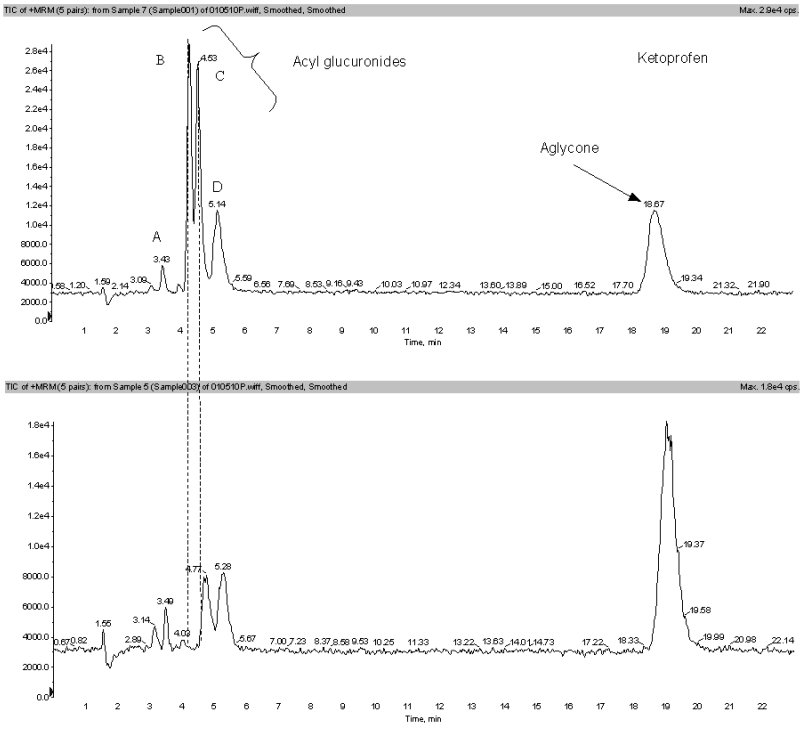
F4.

LC/MS/MS chromatogram for the achiral compound Zomepirac (top) and identification of the 1-O-acyl glucuronide peak following hydrolysis by -glucuronidase (bottom).



F5.

LC/MS/MS chromatogram for the chiral compound Ketoprofen (top) and identification of the 1-O-acyl glucuronide peak following hydrolysis by -glucuronidase (bottom).



T3.
Determination of unmetabolized aglycone and percentage of biosynthesized acyl glucuronides.

Compound	Concentration T_0 (µg/mL) *C.V. (%)*	Concentration T_{4hr} (µg/mL) *C.V. (%)*	Acyl glucuronide formed (%)
Tolmetin	84.6 *3*	79.9 *2.8*	5.6
Zomepirac	120 *5.1*	93 *10.6*	22.5
Suprofen	101 *2.8*	31.7 *2.7*	68.6
Diclofenac	39.3 *6.3*	4.2 *9.8*	89.4
Fenoprofen	125 *7.9*	55.3 *8.6*	55.8
Ibuprofen	53.3 *10.7*	24.9 *5.8*	53.3
Ketoprofen	72.3 *13.7*	26.9 *13.3*	62.8
Furosemide	110 *1.2*	88.9 *2.8*	19.2

T4.
Relative proportions of acyl glucuronides after four hours (other isomers are acyl migrated isomers of 1-O- acyl glucuronide but their exact chemical structure have not been determined).

	Percentage of Acyl glucuronides			
Compound	**1-O-β**	**Other isomers**		
Tolmetin	26 (B)	38	33	3
Zomepirac	48 (B)	24	19	9
Suprofen	54 (C)	37	8	0.4
Diclofenac	26 (B)	62	12	
Fenoprofen	63 + 29 (B+C)	8		
Ibuprofen	≈ 100 (C)			
Ketoprofen	42 + 39 (B+C)	14 5		
Furosemide	≈ 100			