Ambrosio G, Botrè F, de la Torre X, Mazzarino M

In vitro investigation on the effect of antifungals, benzodiazepines and non-steroidal anti-inflammatory drugs on the metabolic profile of morphine

Antidoping laboratory, Federazione Medico Sportiva italiana, Rome, Italy

Abstract

We evaluated the ability of non-prohibited agents (benzodiazepines, antifungals and non-steroidal anti-inflammatory drugs) to alter phase II metabolic profile of morphine by *in vitro* assays.
Morphine was incubated with human liver microsomes (or recombinant uridine diphosphoglucuronosyl-transferases) in the absence and in the presence of the selected inhibitors. The amount of morphine in both free and glucuronated fraction was determined by liquid chromatography coupled with low resolution mass spectrometry after dilution with an aqueous solution of the corresponding deuterated internal standards.
Our results show that the *in vitro* protocol developed in this study is able to reproduce the *in vivo* phase II metabolism of morphine. Morphine glucuronidation is principally carried out by UGT2B7 isoform and minimally by the UGT2B4, UGT2A8 and UGT2A9 isoforms. The inhibition studies, carried out using the *in vitro* protocol set up in this study, show that the activity of the isoform UGT2B7 is significantly reduced in the presence of antifungals (miconazole, ketoconazole, itraconazole, fluconazole), triazolam and ibuprofen. No appreciable modifications were, instead, registered in the presence of the others agents considered in this study.

Introduction

The WADA has banned the use of morphine only in competition, with a threshold value of its urinary concentration set at 1000 ng/mL [1,2]. Morphine is extensively metabolized by liver mainly via conjugation with glucuronic acid, catalyzed by uridine glucuronosyl-transferases [3]. Both physiological and non-physiological factors might alter the pharmacokinetics of morphine, and therefore its urinary levels. Among those non-physiological factors, a very important role is represented by drug-drug interactions. Little information is available regarding the relevance of this phenomenon in doping control field.Here we have investigated the metabolism of morphine by in vitro assays in the absence and in the presence of agents reported in literature to be inhibitors of the UGTs involved in the glucuronidation of morphine [4].

Experimental

Chemicals and reagents
Morphine, morphine-3-glucuronide, morphine-6-glucuronide and their deuterated analogues were from Cerilliant (Sigma-Aldrich, Milano, Italy). The inhibitors and all the chemicals were supplied by Sigma-Aldrich (Milano, Italy).
The reagents for the in vitro studies and the enzymatic proteins were purchased from BD Biosciences (Milano, Italy).

In vitro protocol
The incubation mixtures (total volume 250 µL) contains 0.1 M sodium phosphate buffer (pH 7.4), the substrate in DMSO at final concentration of 10 µM, 8 mM magnesium chloride, 25 µg/mL alamethicin and 2mM cofactor Uridine 5'-Di-Phospho-α-D-glucuronic acid. Samples were pre-warmed at 37 °C for 5 minutes and HLM (0.5 mg/mL) or c-DNA expressed UGT isoforms (0.5 mg/mL) were added and the reaction was started. After incubations for 30 minutes at 37 °C,

 ISBN 978-3-86884-040-7

250 µL of ice-cold acetonitrile were added to stop reactions; and the samples were centrifuged at 12000 rpm for 5 minutes. One sample (negative control) containing all reaction mixture components except proteins was added for each substrate.

Analytical procedure
Sample pre-treatment includes a dilution step with an aqueous solution of the deuterated internal standards at a final concentration of 100 ng/mL.
The chromatographic separation was performed using an Agilent 1100 (Agilent Technologies S.p.a, Milano, Italy), a C18 (150 X 2.1 mm, 5 µm) column from Supelco (Milano, Italy) and bi-distilled water (A) and acetonitrile (B) both containing 0.05% of formic acid as mobile phases. The chromatographic separation was carried out at 30 °C using 98% of A for seven minutes. The flow rate was set at 300 mL/min.
The mass spectrometry was an API3000 triple-quadrupole system (Monza, Italy) with negative electrospray ionization. The data were acquired using selected reaction monitoring (morphine: *m/z* 286/165, CE 35 eV; morphine glucuronide: *m/z* 462/286, CE 30 eV; morphine deuterated: *m/z* 289/165, CE 35 eV; morphine glucuronide deuterated: *m/z* 465/289, CE 30 eV).

Results and Discussion

The optimal mass spectrometry conditions were obtained by infusion of morphine and morphine glucuronide solutions at a concentration of 10 µg/mL in negative ionization mode. For all compounds only the deprotonated molecular ion $[M-H]^-$ was observed in the MS spectra. Product ion experiments were then performed at different collision energies (20, 30 and 40 eV) in order to obtain diagnostic fragments. The ion transitions selected for each compound and the relative collision energy are reported in the experimental part.
For the chromatographic conditions different stationary phases (C8, C18 and HILIC), mobile phase additives (formic and acetic acid, ammonium formate and acetate) and column temperatures (30, 40 and 60 °C) were tested. Satisfactory results in term of reproducibility, sensitivity, peak shape, selectivity, chromatographic retention and time of analysis were obtained using a C18 column and the chromatographic conditions reported in the experimental section (Figure 1A).
Concerning the results obtained by the *in vitro assays,* the protocol developed in this study is able to reproduce the *in vivo* phase II metabolism of morphine (50-60% is metabolized at M3G, 9-10% at M6G and l'8-10% unchanged) (Figure 1A) [3]. The glucuronidation of morphine is mainly catalyzed by the UGT2B7 isoform. The UGT1A8, UGT1A9 and UGT2B4 isoenzymes are involved minimally; whereas the others isoforms tested were not involved in the formation of morphine glucuronide (see Figure 1B).
The inhibition studies show that the catalytic activity of the isoform UGT2B7 is significantly reduced in the presence of miconazole, ketoconazole, triazolam and ibuprofen (Figure 2 and Table 1). No appreciable modifications were, instead, registered in the presence of the others agents considered in this study.

 ISBN 978-3-86884-040-7

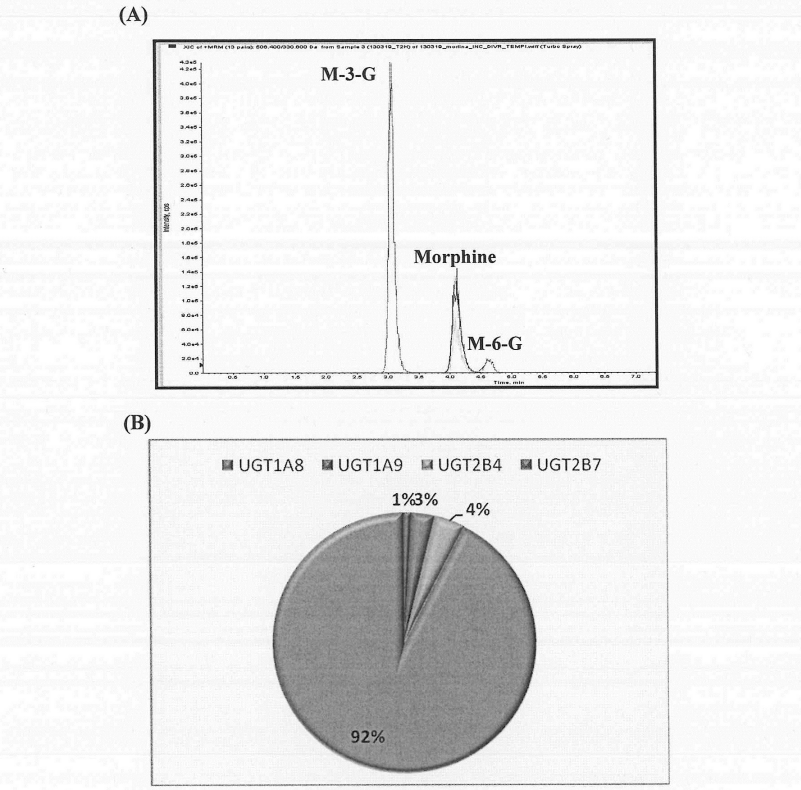

Figure 1: *In vitro* metabolic profile of morphine (10 µM) resulting from the *in vitro* protocol set up in this study and reported in the experimental part **(A)**; characterization of the UGT isoforms involved in the morphine glucuronidation resulting from the *in vitro* protocol set up in this study and reported in the experimental part **(B)**.

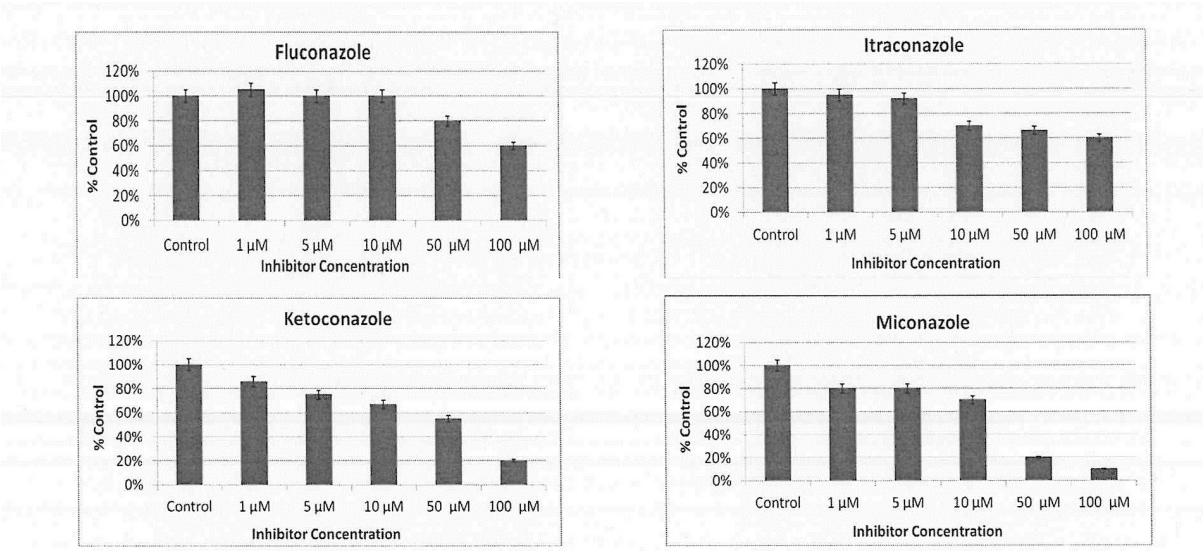

Figure 2: Alteration of the catalytic activity of the UGT2B7 (the most involved isoform in the phase-II metabolism of morphine) in the presence of different concentration (1, 5, 10, 50, and 100 µM) of the antifungals considered in this study. Each point represents the mean value of three independent determinations± the deviation standard of the analytical method.

ISBN 978-3-86884-040-7

Class of inhibitors	Inhibitors	Inibitors concentration (µM)				
		1	5	10	50	100
NSAIDs	Diclofenac	-	-	-	-	-
	Ibuprofen	-	-	++	++	++
	Ketoprofen	-	-	-	-	-
	Nimesulide	-	-	-	-	-
Benzodiazepines	Alprazolam	-	-	-	-	-
	Bramazepam	-	-	-	-	-
	Clonazepam	-	-	-	-	-
	Lorazepam	-	-	-	-	-
	Lormetazepam	-	-	-	-	-
	Triazolam	-	++	+++	++++	++++

- no inhibition; ++ inhibition higher than 20 %, +++ inhibition higher than 50%, ++++ inhibition higher than 70%

Table 1: Alteration of the catalytic activity of the UGT2B7 (the most involved isoform in the phase-II metabolism of morphine) in the presence of different concentration (1, 5, 10, 50, and 100 µM) of the NSAIDs and benzodiazepines considered in this study.

Conclusions

The data obtained from the *in vitro* investigations clearly show that inhibitory drug-drug interactions at the level of drug phase II metabolism may occur between morphine and antifulgals, benzodiazepines and non-steroidal anti-inflammatory drugs causing alterations of the kinetics of biotransformation and excretion of the metabolites selected as markers of drug intake. This in case of threshold compounds might cause “false negative” results due to the reduced levels in urine.
In future we plan (i) to monitor the real occurrence of antifungals, benzodiazepines and non-steroidal anti-inflammatory drugs in the samples analyzed by the WADA-accredited anti-doping laboratories, and (ii) to confirm our *in vitro* observation by conducting *in vivo* studies.

References

[1] World Anti-Doping Agency. The 2014 Prohibited List. International Standard, Montreal(2014) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2014_EN.pdf (access date 20.06.2014).
[2] World Anti-Doping Agency. Decision Limits for the confirmatory quantification of threshold substances, (WADA Technical Document TD2013DL) (also available on-line, at the website www.wada-ama.org).
[3] Taylor K , Elliott S. (2009) A validated hybrid quadrupole linear ion-trap LC–MS method for the analysis of morphine and morphine glucuronides applied to opiate deaths Forensic Science International. 187, 34–41
[4] Takeda et al. (2006) Inhibition of UDP- glucuronosyltransferase 2B7-catalyzed morphine glucuronidation by ketoconazole: dual mechanism involving a novel non competitive mode Pharmacology and Experimental Therapeutics 34, 1277–1282.

Acknowledgements

We acknowledge the World Anti-Doping Agency for the financial support for this research project (project code: 12C17MM).