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Development of an in vitro methodology in metabolite production and analytical techniques: beta-blockers and TOF-MS screening of unknown metabolites

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Abstract

The aim of the project was to develop flexible *in vitro* procedures which can be applied in order to study and predict the metabolic patterns of beta-blockers, and to propose the most prominent target compounds for doping control purposes. The pilot study was carried out with a model compound, propranolol. The correlation between *in vitro* metabolism of enzyme preparations and *in vivo* authentic excretion study samples, and subsequently, the applicability of the *in vitro* model for prediction of metabolic pathways was evaluated. Based on the available literature, the main metabolic routes of beta-blockers involve hydroxylation as phase-I reaction and conjugation to glucuronic acid as prevailing phase-II reaction. Along the soft ionisation techniques in atmospheric pressure, such as electrospray ionization (ESI), direct detection of glucuronide-conjugates is of great interest. The applicability of liquid chromatographic–time-of-flight mass spectrometric (LC-TOFMS) methodology to doping control has been shown in several earlier studies, but in this work the suitability was tested also for the metabolic studies. Finally, analytical experience gained in metabolic studies will be exploited in LC-MS/MS methods in routine doping control.

Introduction

Beta-blockers inhibit adrenergic stimulation of beta-receptors and could be performance-enhancing in those sports which require minimal tremor, e.g. shooting. This group is prohibited in particular sports (P2) by WADA [1]. *In vivo* metabolic studies are time-consuming because of legislation aspects. Alternative pathways are *in vitro* applications, which may utilize e.g. cytosolic or microsomal preparations as source of metabolising enzymes. This *in vitro* approach is well-implemented in drug discovery and also in doping control [2,3] because the assays are straightforward, fast and amenable for experienced staff. In this project we aimed at developing *in vitro* procedure to study the metabolism of beta-blockers, and to propose target compounds for doping control purposes. The model compound of the pilot study was propranolol.

Experimental

In vitro assays for phase-I and phase-II reaction based on our earlier studies [2,3] and consisted of the factors summarised in Table 1. The total incubation volume was 200 µL. Phase-I reaction was initiated with NADPH and the first assay was carried out in water bath (37°C, 6 hrs). UDPGA was then added to enable the subsequent glucuronidation (37°C, 20 hrs). Reaction was stopped by ice-cold acetonitrile (100 µL), samples were centrifuged (5 min, 10 000g), and the supernatants were extracted with ethylacetate in neutral and basic (pH 9.6) conditions. Excretion urine sample (1 x 20 mg propranolol, p.o., 0-4 hrs fraction) was used to evaluate *in vitro-in vivo* correlation of the assay, and processed similarly to *in vitro* samples. Following the ethylacetate extraction, the organic layer was evaporated to dryness and reconstituted in 50 µL of solvent environment which corresponded to the initial state of LC-gradient. The analysis was carried out in positive ion mode electrospray ionisation with UPLC/TOFMS method for which the details have been described earlier in detail [4].

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Reagent	Concentration	Provider
Propranolol (as free base)	*100 µM*	*Sigma*
NADPH	*5 mM*	*Roche*
UDPGA	*5 mM*	*Sigma*
Saccharic acid -1,4-lactone	*5 mM*	*Sigma*
Protein (S9 and microsomal fraction)	*2 mg/ml*	*BD Gentest*

Table 1: Components of the in vitro assay.

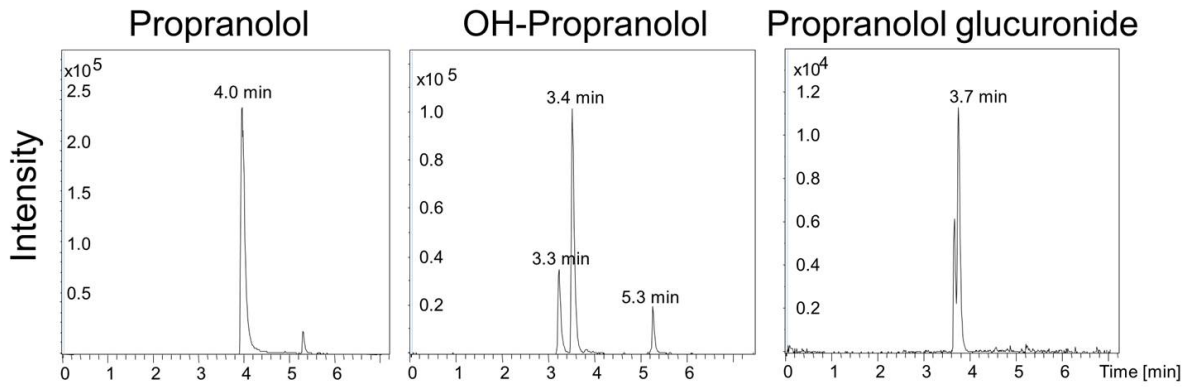

Figure 1: Extracted ion chromatograms of propranolol (m/z 260.1645), hydroxypropranolol (m/z 276.1594), and propranolol glucuronide (m/z 436.1973) from an in vitro enzyme assay.

Results and Discussion

The main reported metabolic routes of propranolol include hydroxylation, dihydroxylation and glucuronide conjugation [5]. In this *in vitro* assay three major peaks of mono-hydroxylated propranolol (Figure 1 and Table 2) and only traces of dihydroxylated propranolol were observed. The main *in vitro* product was identified against reference material as 4-hydroxy-propranolol (t_r 3.4 min), and for the two most abundant others, which were not found in the early fraction excretion urine sample of this experiment, the proposed structures are 5-hydroxypropranolol (t_r 3.3 min) and 7-hydroxy-propranolol (t_r 5.3 min). In phase-II simulation,the parent propranolol and traces of 4-hydroxy-propranolol were detected as glucuronide-conjugates. Propranolol and 4-hydroxypropranolol, in free and glucuronide-conjugated fraction, were the observed *in vivo* metabolites of the excretion study sample. The applied *in vitro* assay was based on the human liver enzyme preparations (S9 and microsomal fraction) and according to these results the assay is capable of producing not only the relevant *in vivo* metabolites of propranolol but also additional, mainly hydroxylated metabolites. The amounts synthesised in one single 200 µL incubation were enough e.g. for qualitative method development purposes. The selected chromatographic conditions were fit for the *in vitro* assay purpose and provided baseline separation of the three mono-hydroxylated metabolites within a total analysis time of 7 minutes. All the metabolites were detected in positive ion ESI mode as protonated molecules $[M+H]^+$ and the maximum measured mass error in the TOFMS was < 3.5 mDa. The analytical set-up consisting of UPLC separation and high resolution MS-analysis of TOF was proven applicable to the screening of metabolic simulations, especially both phase-I and intact phase-II (glucuronide-conjugated) metabolites could be measured in one analytical run.

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Conclusions

Using propranol as model compound the earlier-developed *in vitro* assay provided a fast and straightforward simulation also for beta-blocker metabolism. The metabolites formed in the enzyme-driven process correlated well with earlier published data describing the human metabolism, and can be used in for method development purposes in doping control. Combined with a simple sample preparation process the UPLC/TOFMS-based analytical method offered powerful chromatographic separation and high accuracy mass spectrometric detection for the screening of unknown phase-I and phase-II drug metabolites in one single analytical run.

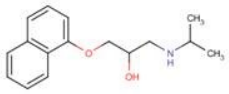
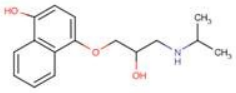
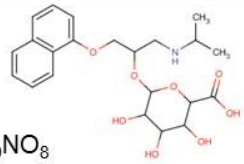
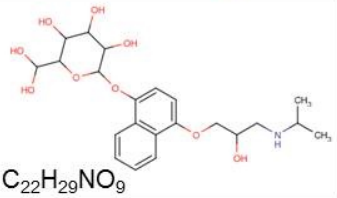
Compound	Formula	Calculated mass	Measured mass	Retention time (min)
Propranolol	 $C_{16}H_{21}NO_2$	260.1645	260.1612	4.0
4-Hydroxy-propranolol	 $C_{16}H_{21}NO_3$	276.1594	276.1590	3.3
Propranolol glucuronide	 $C_{22}H_{29}NO_8$	436.1973	436.1985	3.7
4-Hydroxy-propranolol glucuronide	 $C_{22}H_{29}NO_9$	452.1915	452.1932	1.3

Table 2: Identification and accurate mass measurement of propranolol and metabolites observed in the in vitro enzyme assay.

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