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Importance of phase II metabolites for the detection of beta-blockers by direct urine analysis

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Abstract

By analyzing the excretion urine samples using the “dilute and shoot” approach it was shown that several β-blockers such as carvedilol, propranolol, alprenolol, nebivolol, betaxolol and oxprenolol are subject to extensive phase II metabolism leading to the formation of glucuronic acid conjugates. These conjugates can be assayed in their intact form using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Incorporation of glucuronide metabolites of carvedilol, propranolol, alprenolol and nebivolol into the method is necessary to avoid false negatives in doping control analysis when the “dilute and shoot” approach is used.

Introduction

The chemically diverse class of β-blockers can be detected in urine by GC-MS after derivatization [1], but LC-MS(/MS)-based methods were later demonstrated to be more suitable [2,3]. Regardless of the detection method, sample preparation may or may not include the enzymatic hydrolysis and use solid phase extraction or different organic solvents. From the viewpoint of doping control laboratories, a reliable analytical method is required that would be as simple as possible and also exclude the possibility of false-negatives which could originate from the low recovery or extensive metabolism. Taking into account a relatively high minimum required performance level for β-blockers [4], we have applied direct urine analysis because the sensitivity of modern mass spectrometers should be adequate for this purpose.

Experimental

Urine samples were processed as follows: 200 µL of urine were diluted with 800 µL of solvent mixture (0.1% formic acid and 3% methanol in water with bupranolol as internal standard), and centrifuged at 14000 *g* for 10 min before transfer to a glass vial.

LC–MS/MS analyses were accomplished on an Acquity LC (Waters, Milford, MA, USA) coupled to a TSQ Vantage (ThermoFisher Scientific, San Jose, CA, USA). A Waters Acquity BEH C18 column (100 mm × 2.1 mm, particle size 1.7 µm) maintained at 60°C and protected by a Vanguard BEH C18 column (20 mm × 2.1 mm) was used for separation. Injection volume was 5 µL. The mobile phase flow rate was set to 0.35 mL/min. The elution program started from 0.5-min isocratic step at 95% of 0.1% formic acid in water (A) and 5% of 0.1% formic acid in methanol (B) followed by linear increase to 95% of B within 4.5 min, hold at 95% of B for 2.5 min and then re-equilibration until the end of analysis (10 min).

The heated electrospray ion source was used for ionization. Positive ions were detected in fullscan, product ion scan (during method development) and selected reaction monitoring (SRM) modes. The collision gas pressure was 0.2 Pa (or 1.5 mTorr, argon 99.9995%). Sheath and aux gas pressure (nitrogen from a nitrogen generator, 99.9% purity) was set at 55 and 35 arbitrary units, respectively. The vaporizer and capillary temperatures were set at 370 and 300°C, respectively, with a spray voltage of 4000 V. In the MS/MS experiments the collision energy (CE) was ramped from 5 to 40 eV to select the optimal value for every transition. The mass isolation widths for Q1 and Q3 were set to 0.7 and 1.0 Da (full width at half maximum, FWHM), respectively.

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Results and Discussion

Initially, we have set up a method incorporating all parent compounds enlisted in the WADA prohibited list [5], and it was validated to have the limits of detection at least one tenth of current MRPL [6] stipulated for β-blockers (500 ng/mL). However, after the analysis of real post-administration urines from our reference collection as well as miscellaneous EQAS samples it was found that several β-blockers, *e.g.* carvedilol, propranolol, alprenolol, and nebivolol, could hardly be detected as parent compounds by "dilute and shoot" approach due to their low concentration in excretion urine.
Further, we were successful in identifying the glucuronide metabolites of the abovementioned β-blockers directly in diluted urine in the amount being at least 10 times higher than the unchanged parents. The SRM transitions used in our method are given in Table1.

Name	SRM	CE, V
carvedilol	407 > 224 407 > 100	22 30
propranolol	260 > 183 260 > 155	18 26
alprenolol	250 > 173 250 > 91	18 35
carvedilol glucuronide	583 > 407 583 > 222	25 35
propranolol glucuronide	436 > 260 436 > 155	25 40
alprenolol glucuronide	426 > 250 426 > 116	25 35
oxprenolol glucuronide	442 > 266 442 > 72	35 25
betaxolol glucuronide	484 > 308 484 > 116	25 35
nebivolol glucuronide	582 > 406 582 > 151	32 35

Table 1. Summary mass spectral data for selected β-blockers and their glucuronide metabolites.

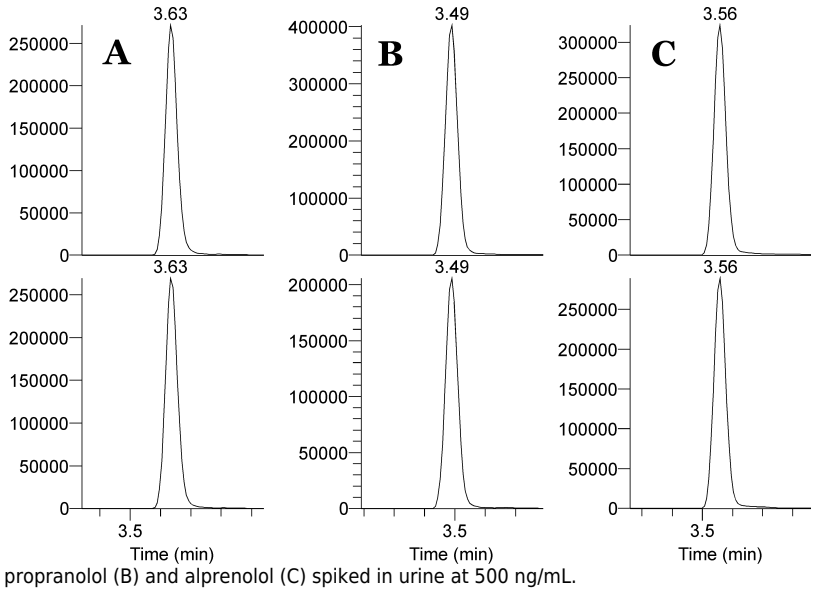

Fig. 1. Carvedilol (A), propranolol (B) and alprenolol (C) spiked in urine at 500 ng/mL.

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Interestingly, in most cases (except for 4-hydroxyalprenolol – data not shown) the glucuronides were detected as two well resolved chromatographic peaks probably related to the different glucuronidation sites (*O*- and *N*-glucuronides). While some of the glucuronide metabolites are currently available from Toronto Research Chemicals, Inc., at the moment of performing this study we did not have them at hand.
The chromatograms for blank urine sample spiked with carvedilol, propranolol and alprenolol at 500 ng/mL, and for excretion urines obtained after administration of these compounds are shown in Fig.1 and 2, respectively.

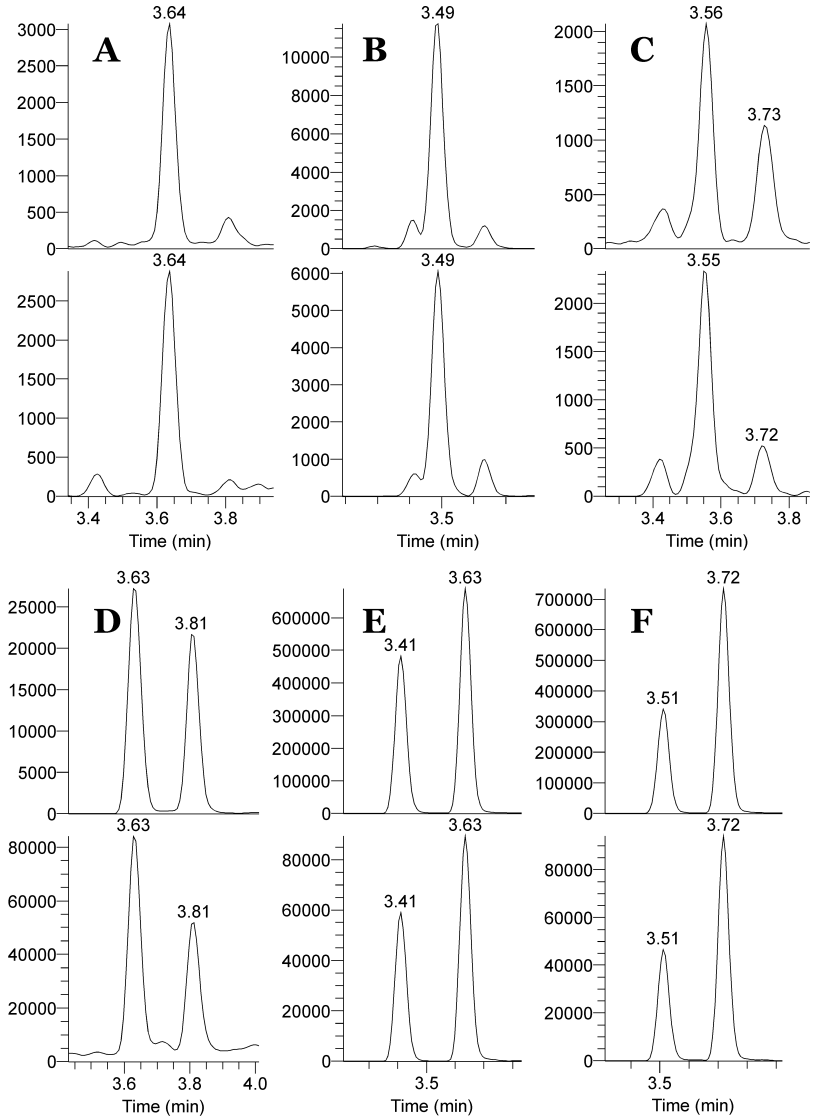

Fig. 2. Carvedilol (A), propranolol (B), alprenolol (C) and respective glucuronides (D, E, F) in real post-administration urines.

It is clearly seen that parent drugs are detectable at low ng/mL level approaching the limit of detection, while incorporation of the glucuronide metabolites makes the method reliable and fit-for-purpose.
In addition, other valuable glucuronide metabolites are shown in Fig. 3.
While being well detectable in excretion urines as parent compounds, oxprenolol and betaxolol are subject to extensive glucuronidation as the abundance of respective glucuronide metabolites is *ca*. 10 times higher.

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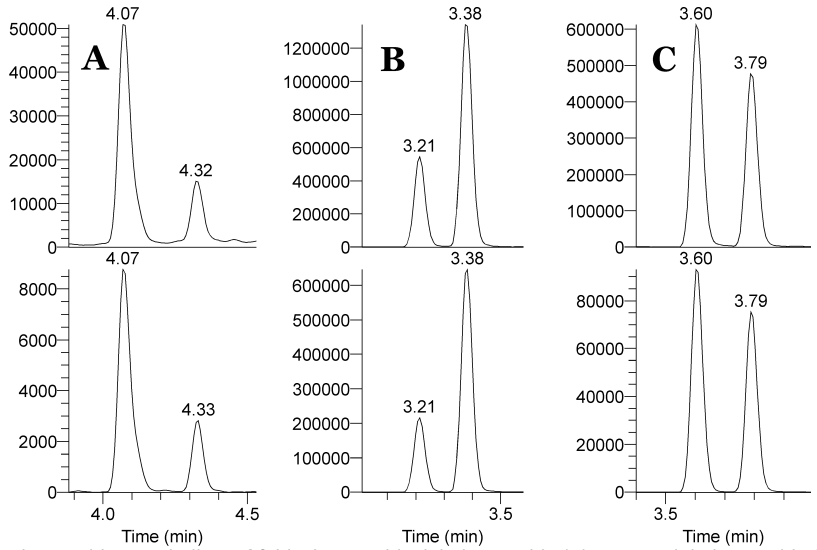

Fig. 3. Other valuable glucuronide metabolites of β-blockers: nebivolol glucuronide (A), oxprenolol glucuronide (B), and betaxolol glucuronide (C).

Conclusions

The critical importance of phase II metabolites for the determination of β-blockers by the "dilute and shoot" approach was demonstrated. The inclusion of glucuronides into the method provides a reliable and express solution to detect β-blockers in urine at the levels being fully compliant with the WADA technical guidelines. To assure full compliance with the upcoming MRPL for β-blockers (100 ng/mL), injection volume might have to be increased to 10 µL. However, a complete validation of the method will depend on availability of synthetic reference materials of β-blocker glucuronides.

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