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Screening for N-desmethyl- and N-bisdesmethyl metabolites of Sibutramine using liquid chromatography-tandem mass spectrometry

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Introduction

Since January 2006, the list of prohibited substances established by the World Anti-Doping Agency includes the antidepressant / anti-obesity drug Sibutramine. Due to its rapid degradation to its active metabolites N-desmethyl and N-bisdesmethyl Sibutramine, reference compounds were synthesized and included into an existing screening assay to allow the unambiguous determination of these metabolic products in human urine using liquid-liquid extraction followed by liquid chromatography – tandem mass spectrometry. Characteristic product ions, obtained after electrospray ionization and collision-induced dissociation, were elucidated using high resolution / high accuracy mass measurements with a hybrid linear ion trap / orbitrap mass analyzer.

Experimental

Sample preparation [1].

To 3 combined aliquots of 2.5 mL of urine, 1250 ng of the ISTD d5-isoxsuprine, 100 mg of cysteine and 0.45 mL of HCl (10M) are added. The sample is mixed thoroughly and incubate for 45 min at 80°C. Five mL of *tert*.-butylmethyl ether is added to the urine specimen, the sample is shaken mechanically for 20 min and centrifuged for 5 min. The ether layer is discarded. Then, a volume of 0.9 mL of KOH (5M), 200 mg of K_2CO_3 / $NaHCO_3$ (2:1,w/w), 1 ml of *tert*.butanol, 3 g of anhydrous sodium sulfate and 5 mL of *tert*.-butylmethyl ether are added. The sample is shaken for 20 min and centrifuged for 5 min. The ether layer is transferred to a glass tube and concentrated. Then, 100 µL of 0.06 N HCl is added and an aliquot is transferred for LC-MS/MS analysis.

LC-MS/MS parameters.

LC-MS/MS:	Applied Biosystems API 2000
Ion source:	APCI, 400°C

Ionization-/Acquisition-mode:	positive / MRM
Collision gas:	Nitrogen, 1.8e-5 torr
Column:	Nucleodur C18 , 4x70mm, particle size 5µm (Pyramid)
Eluents / Flow:	A=5mM ammonium acetate in H_2O, 0.1% acetic acid, pH=3.5
	B=acetonitrile / 0.8 mL/min
Gradient:	isocratic (100% A) 1 min, linear gradient to 100% B in 8 min, re-equilibration at 100% A for 2.6 min

Results

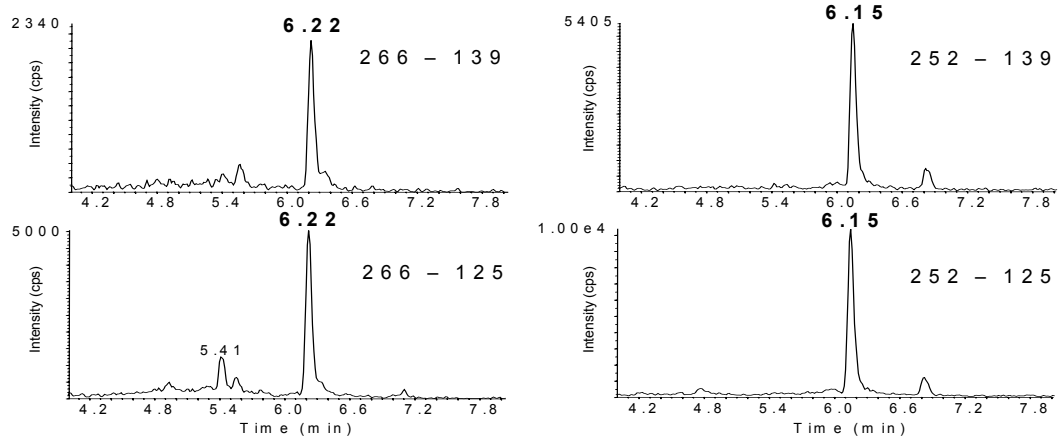


Figure 1: Extracted ion chromatograms of an excretion study urine sample obtained from a female patient being treated with Sibutramine hydrochloride monohydrate (Reductil®). Both metabolites of Sibutramine are detected using respective precursor / product ion transitions at 266-125 and 252-125.

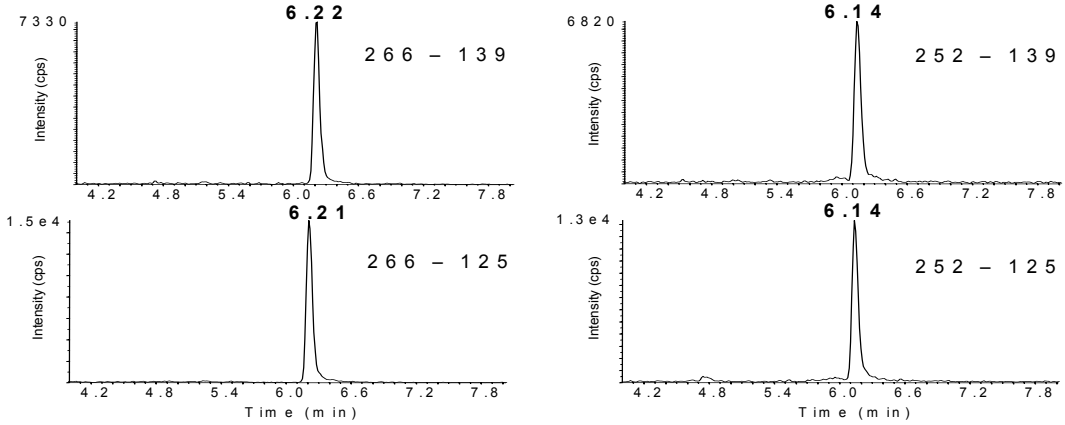
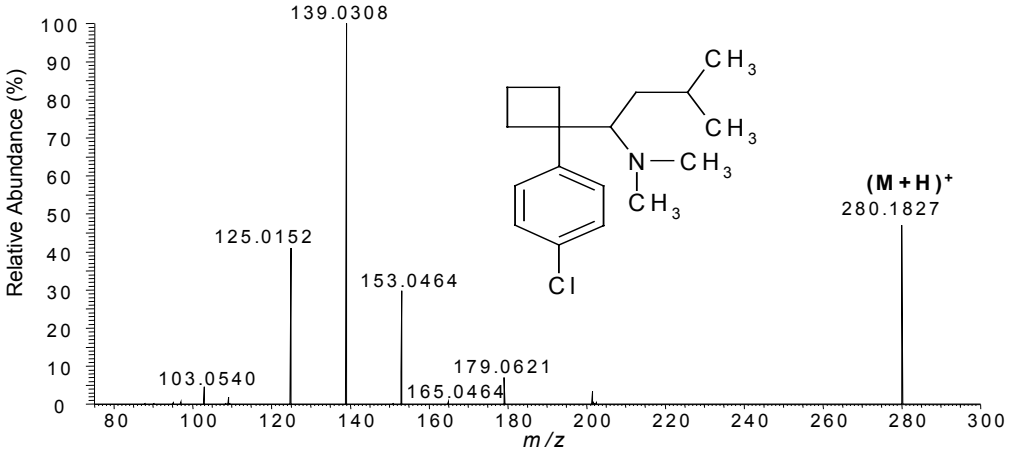
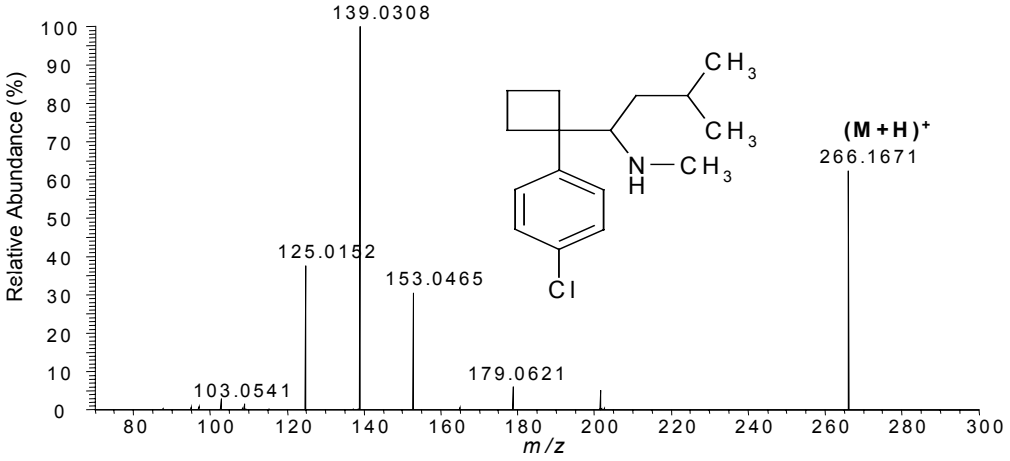


Figure 2: Extracted ion chromatograms of a urine sample spiked to 50 ng mL^{-1} of both active metabolites of Sibutramine using respective precursor / product ion transitions at 266-125 and 252-125.

a)



b)



c)

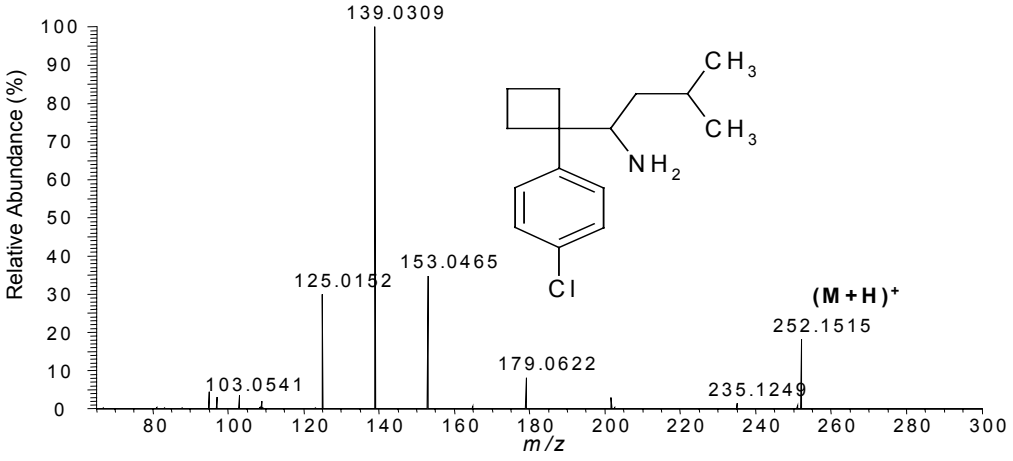


Figure 3: ESI-product ion spectra of protonated molecules of a) Sibutramine (mol wt = 279, normalized collision energy = 25); b) desmethyl Sibutramine (mol wt = 265, normalized collision energy = 22); and c) bisdesmethyl Sibutramine (mol wt = 251, normalized collision energy = 22).

Based on diagnostic product ions, the extended screening procedure was validated for both Sibutramine metabolites using a triple quadrupole mass spectrometer. Items such as lower limits of detection (6-40 ng mL^{-1}), recoveries (39-42%), intraday precision (low: 5.5-10.6%, medium: 4.9-5.9%), high: 12.8-16.4%) and interday precision (low: 15.0-22.8%, medium: 17.7-18.6%), high: 16.5-25.6%) were evaluated, and a clinical spot urine sample was analyzed to demonstrate the applicability of the developed assay in sports drug testing [2].

Conclusion

The characteristic dissociation pathways of the metabolites of Sibutramine after ESI and CID allowed their implementation into a fast and sensitive routine assay in doping control analysis. The method has proven suitable specificity and sensitivity as no interfering signals were observed in blank urine specimens, and target compounds were detected in spiked as well as excretion study urine samples. According to published data on the metabolism of Sibutramine[3], both metabolites are not conjugated to phase-II-metabolites before renal elimination. Hence, a sample preparation would not require any hydrolysis step such as the treatment with aqueous hydrochloric acid at elevated temperatures, but due to the inclusion of the desmethylated Sibutramine metabolites into an existing screening procedure, the entire method was to apply to these new target analytes.

References

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