QUALITATIVE DETERMINATION OF FUROSEMIDE IN ATHLETES URINE BY UHPLC-MS/MS

Azamjon B. SOLIEV, Azimjon A. MAMADRAHIMOV, Noiba M. RAKHIMOVA, Nail J. SAGDIEV,
The Republican Scientific Practical Centre for Sports Medicine at the National Olympic Committee of Uzbekistan

Rezumat

Determinarea calitativă a furosemidului în urina sportivilor prin metoda HPLC-SM/SM

A fost elaborată o metodă sensibilă şi simplă de determinare a furosemidului în urina sportivilor prin metoda HPLC – spectrometria de masă. Au fost optimizate condiţiile acestei metode. Metoda elaborată este utilizată cu succes pentru determinarea furosemidului în urina sportivilor ca un compus doping.

Cuvinte-cheie: *HPLC, spectrometru de masă, furosemid, testare doping*

Резюме

Качественное определение фуросемида в моче спортсменов методом УВЭЖХ-МС/МС

Разработан чувствительный, селективный и простой метод определения фуросемида в моче спортсменов методом Ультра высокоэффективной жидкостной хроматографии масс-спектрометрии (УВЭЖХ-МС/МС). Были оптимизированы условия как жидкостной хроматографии, так и масс-спектрометрии. Разработанный метод был успешно использован для определения фуросемида в моче спортсменов как допинговое соединение.

Ключевые слова*: УВЭЖХ, масс-спектрометр, фуросемид, допинг-тест*

Introduction

It is well known that furosemide is used as a diuretic in medicine. Due to this property, the athletes who compete in weight class sports such as boxing, wrestling and weightlifting, effectively use it to reduce the body weight, and also as a masking agent to conceal the other prohibited compounds in the body fluids by ample urination [1, 2]. That is why furosemide and its derivatives are considered as doping compounds and included to the WADA's prohibited list of chemical substances [3]. Diuretics are ranked third (14% positive doping tests) after anabolic steroids (54%) and stimulants (29%) among the prohibited compounds abused by the athletes [1].

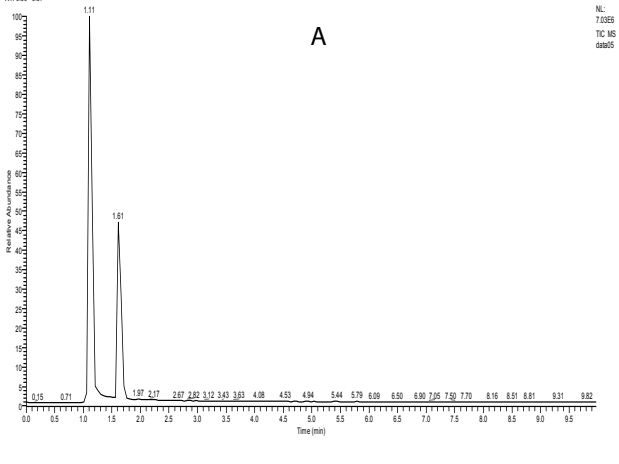
Materials and methods

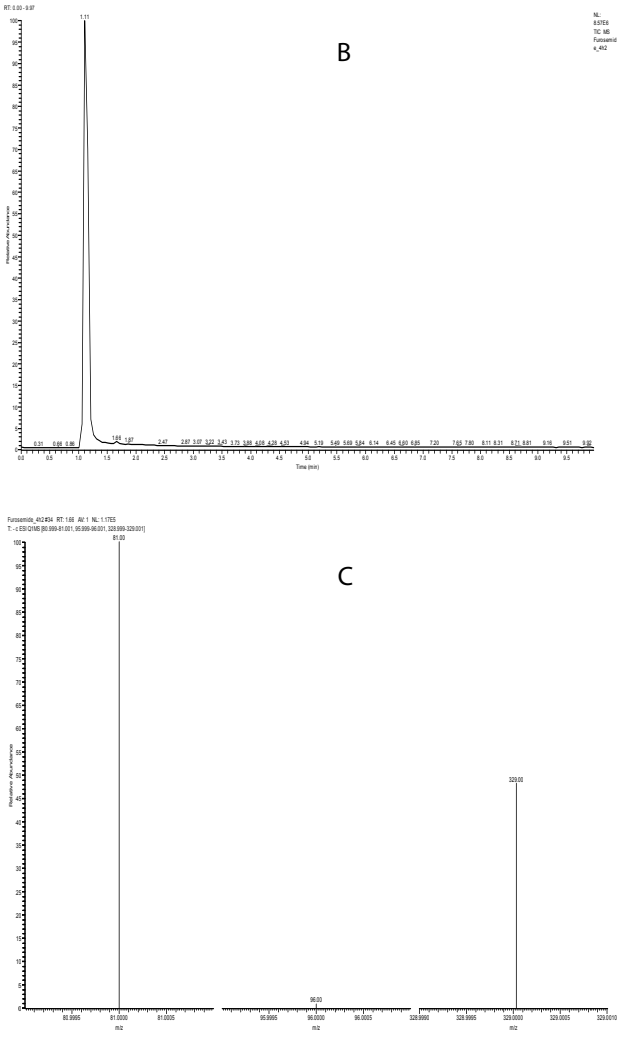
Instrumentation and Chemicals

Ultimate 3000 Quaternary Standard ultra-high performance liquid chromatography tandem TSQ Quantum Access Max triple quadrupole mass-spectrometer (UHPLC-MS/MS) from Thermo Fisher Scientific was used throughout the study. The UHPLC column used was a HyperSil Super Gold C18 (100 mm×2.1 mm, 1.9 µm) (Thermo Fisher Scientific, USA). Furosemide 1% 2 ml (10 µg/µL) aqueous solution and tablets containing 40 mg of active compound produced by the local pharmaceutical company was purchased from the local drug store. HPLC grade acetonitrile was purchased from *Sigma-Aldrich Trading Co* (Schnelldorf, Germany). Orthophosphoric acid and sodium hydroxide (purity >98.0%) was purchased from the local chemical company that specializes on the trade of chemical reagents and equipment. HyperSep Retain PEP SPE cartridges were obtained from *Thermo Fisher Scientific* (CA, USA). Ultrapure water was prepared by a water purification system from *Sartorius Lab Instruments GmbH Et Co. KG* (Goettingen, Germany). All other chemicals were analytical grade and used without further purification.

Sample Preparation

Standard furosemide containing urine sample was prepared by adding 2 ml 1% intramuscular solution (10 µg/µL) of furosemide to 98 ml of urine to make a total of 100 ml. From this 0.75 ml aliquots have been taken into 3 eppendorf tubes to add the equivalent amounts of water, 3% solution of orthophosphoric acid and 3% solution of sodium hydroxide solution, respectively. The samples were applied into HyperSep Retain PEP SPE cartridges activated with 1 ml of methanol and equilibrated with methanol – water (containing 1% H_3PO_4 solution) – 5-95 v/v. The analytes were eluted out with 0.75 ml of pure methanol. The obtained samples were then dried by a vacuum concentrator and reconstituted in 1 ml of mobile phase containing acetonitrile – water in a volume ratio of 35:65 (v/v) and passed through 0.2 µm Millipore filter. 1 µL of the samples were injected into UHPLC-MS/MS equilibrated in the above mentioned mobile phase in an isocratic elution mode.





*The UHPLC-MS/MS analysis of urine sample. (**A**) liquid chromatography profile of furosemide containing standard urine sample; (**B**) liquid chromatography profile of urine sample collected after taking furosemide tablet; (**C**) mass-spectrometry profile of urine samples containing furosemide. The presence of furosemide was confirmed with its characteristic negative ion m/z 329 shown in negative SIM mode.*

Results and discussions

Many existing procedures for the determination of diuretics in literature outline quite complex and expensive methods. In doping control, diuretics extraction from urine includes multistep treatment with rare chemicals, following by chromatographic separation and data interpretation of the obtained spectral results. The lack of expensive rare chemical reagents and equipments as well as time-consuming tedious methods led us to modify more reliable and cheaper ways of analysis of drugs that is abused by the athletes. Therefore, the main goal of this research was to simplify the analysis of furosemide in urine.

Initially, for the method development, 100 ml of standard urine samples both containing furosemide and without furosemide were prepared *in vitro* (the ultimate concentration of furosemide in furosemide containing sample was 200 ng/µl). The *in vivo* urine samples were collected before and after taking 1 tablet of furosemide that contain 40 mg of active compound.

The MS was operated in negative HESI-II mode, with data collected from 1-10 min. Initially, full scan Q1 mass spectra was acquired over the range of *m/z* 50-1500 then SIM mode was applied for qualitative analyses of furosemide in urine. The SIM mode was more sensitive in comparison with full scan mode in selection of ions. It is because in the last one, typically, two transitions precursor-products are only monitored that can mostly give sufficient information to define the necessary compound. In our research, the SIM mode was also successfully applied in defining furosemide parent ion from other concomitant ions having the same m/z present in urine.

Conclusion

The developed method proved the suitability of liquid chromatography-tandem mass spectrometry for the confirmation of the presence of furosemide in urine. The method was found to be selective and reliable towards furosemide, since the other components of urine do not interfere with its detection.

Sample preparation and the analysis times have been reduced and simplified. The reduction of time and optimization of the developed method allowed achieving chromatographic separation of furosemide in short time (within 2 minutes). Selection of diagnostic fragmentation reactions and optimal collision energies allowed to achieve high selectivity in searching the necessary ion fragments.

The developed method was qualitatively validated to be used in anti-doping tests.

References

1. Y. L. Tseng, M. H. Shieh, C. T. Lin et al. *Detection of Diuretics in Urine during Sports Events in Taiwan*. In: Tzu. Chi. Med. J., 2004; nr. 16, p. 69-77.
2. L. Dirikolu, A.F. Lehner, C.G. Hughes et al. *The Detection, Quantification and Pharmacokinetics of Furosemide and its Effects on Urinary Specific Gravity Following Intravenous Administration to Horses*. In: Veterinary Therapeutics, 2003; nr. 4(4), p. 350-363.
3. *2015 List of Prohibited Substances and Methods.* WADA, September 20, 2014.

Azamjon B. Soliev,
6, Almazar str., 100003, Tashkent, Uzbekistan,
E-mail: 1136001@gmail.com
Tel./Fax: (+99871) 245-72-42