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Excretion studies with thiazide diuretics

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Introduction

In sports diuretics are used for two main reasons: to flush previously taken prohibited substancees with forced diuresis and in sports where weight classes are involved to achieve acute weight loss. Therefore diuretics are included on the prohibited list published by WADA[1]. A therapeutical important group of diuretics are the thiazides.
Thiazides can hydrolyse in aqueous media. For altizide (ALT), hydrochlorothiazide (HCT) and chlorothiazide (CLT) a common degradation compound can be formed named 4-amino-6-chloro-1,3-benzenedisulphonamide (ACB) [2, 3].
Despite a comprehensive review on metabolism and elimination of thiazide diuretics information regarding the urinary detection of ACB is not available [4]. Therefore, the aim of this study was to determine excretion times and profiles for ACB in urine after oral administration of HCT and ALT. Besides, the excretion of the parent drug HCT and ALT and the metabolite CLT was also monitored.

Experimental

Chemicals and reagents

HCT and CLT were obtained from Ciba-Geigy (Groot-Bijgaarden, Belgium), ALT from Continental Pharma (Brussels, Belgium). 4-amino-6-trifluoromethyl-1,3-benzendisulponamide (ATFB) (degradation product of bendroflumethiazide) and ACB were purchased from Sigma-Aldrich (Bornem, Belgium). Methanol (analytical-reagent). (MeOH), ammonium acetate (NH_4Ac) p.a, acetonitrile (ACN). and HPLC grade water were from Biosolve (Valkenswaard, The Netherlands). Ethyl acetate (EtOAc) was from Acros (Geel, Belgium) and was distilled before use. Buffer pH 7.0 was obtained by dissolving 35.5 g

disodiumhydrogenphosphate.dihydrate and 7.0 g sodiumdihydrogen phopshate in 500 mL distilled water and pH adjusted with phosphoric acid.

Excretion study

The study was performed with 6 healthy volunteers (1 female and 5 male) aged 23, 28, 29, 31, 34 and 39 weighing respectively 82, 68, 74, 80, 95 and 74 kg. The study protocol was reviewed and approved by the ethical committee of the Ghent University Hospital (UZGent, Project EC UZG 2007-054). Each volunteer signed a statement of informed consent and was administered one tablet Docspirochlor® (Docpharma, Heverlee, Belgium) containing 25 mg HCT and 25 mg spironolactone. Urine samples were collected before (0h) and quantitatively at 1, 2, 3, 6, 9, 12 hours after intake. Additional samples were collected after 24, 36, 48, 72, 96 and 120 h. Four weeks later, the same volunteers were given Aldactazine® (Continental Pharma, Diegem, Belgium),containing 15 mg ALT and 25 mg spironolactone). Sampling was similar as above. All urine samples were stored at -20°C awaiting analysis. Volume and density were measured and all samples were analyzed in duplicate.

Sample treatment

An internal standard (IS) solution (50 µL TFSA, 1 µg/mL) was added to 1 mL of urine, followed by the addition of 1 mL of a 0.1 M phosphate buffer (pH 7.0). Liquid-liquid extraction was performed by rolling for 5 min with 4 mL ethyl acetate. After centrifugation (8000 g) the organic layer was transferred into a new tube and evaporated until dry under oxygen free nitrogen (OFN) at room temperature. The remaining residue was dissolved in 200 µL of the initial mobile phase, 20 µL was injected into the HPLC-system.

Apparatus

The HPLC system consisted of a Surveyor MS-pump and Surveyor autosampler with a 25 µL sample loop (all from Thermo Separation Products, Thermo, San Jose, CA, USA). Analysis was performed on an omnisphere C18 (50x2mm, 3µm) column fom Varian (Sint-Katelijne-Waver, Belgium). The column was maintained at 35ºC. The mobile phase consisted of water (A) and MeOH, both containing 1mM ammonium acetate. Gradient elution at a flow rate of 0.3 mL/min was performed as follows: 95% A for 2 min followed by a decrease to 0% A in 4 min and an increase to the initial condition of 95% A in 0.1 min followed by an equilibration step of 1.9 min before the next injection. Total analysis time per sample was 8 min. The LC

effluent was pumped to a Quantum Discovery mass spectrometer (Thermo, San Jose, CA, USA) equipped with an ESI source, operated in the negative ionisation mode.
The capillary temperature was 350 ºC. The sheath gas flow rate was set at 70 arbitrary units. No auxilliary gas was used. Detailed SRM detection settings are presented in table 1.

Table 1: SRM detection settings for the investigated substances.

	Precursor $[M-H]^-$	TLV	CE	PI
ACB	284	-119	-23	136
			-23	169
ATFB (IS)	318	-116	-25	214
			-33	200
ALT	382	-119	-16	341
			-18	269
CLT	294	-97	-30	214
			-46	179
HCT	296	-119	-21	269
			-24	205

CE: collision energy, PI: product ion, TLV : Tube Lens Voltage

Results

Method validation

Using a least square fit, good linearity ($r^2 \geq 0.998$) was obtained for ACB and HCT in the range 10 – 200 ng/mL and 2.5-50 ng/mL for CLT. For ALT no calibration curve could be constructed and detection was only done qualitatively.
None of the calibration curves were forced through the origin and for the regression calculation a weighing factor of 1/x was used for all data points.
These values did not exceed the maximum tolerances established for repeatability and reproducibility. Deviation of the mean measured concentration from the theoretical concentration (accuracy) for all compounds was below the acceptable threshold of 15 % and 20 % for all levels in the calibration curve range.
Regarding the selectivity, interferences from other doping agents could not be found. In addition analysis of 10 different blank control urine samples did not result in the detection of interfering substances, proving the specificity of the method.
The limit LOQ of the method was 10 ng/mL for ACB and HCT and 2.5 ng/mL for CLT. The limit of detection was 1 ng/mL for ALT, 0.5 ng/mL for ACB and HCT and 0.125 ng/mL for CLT.

Excretion Study

Docspirochlor (25 mg hydrochlorothiazide/ 25 mg spironolactone)

For all volunteers HCT could be detected already one hour after intake (Figure 1).
The maximum urinary concentration of HCT was reached after 6 hours in 5 out of the 6 volunteers. In one subject the peak concentration was reached after 3 hours. Maximum concentrations ranged between 11.9 μg/mL and 17.6 μg/mL.

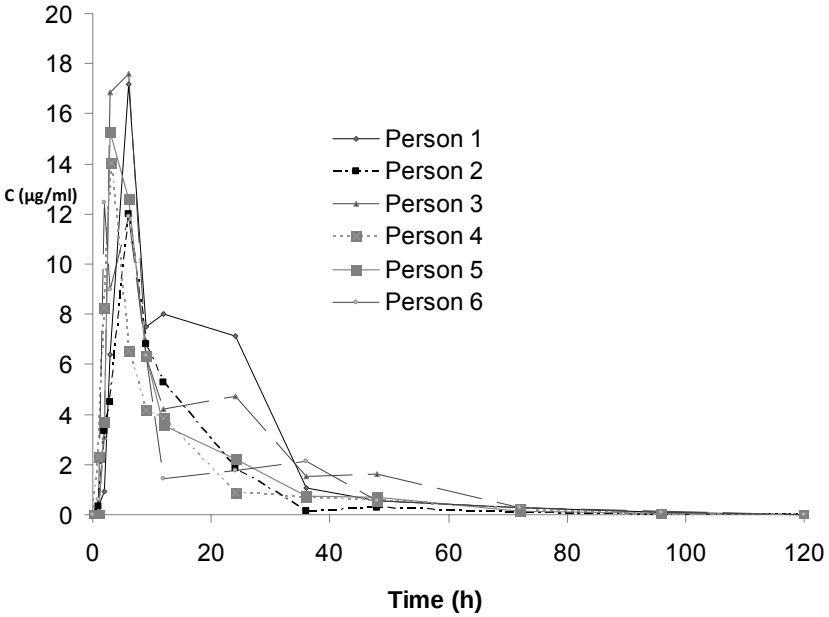

Figure 1: Urinary excretion profiles for HCT after intake of 25 mg.

HCT could still be detected when the urine-collection was finished (120h). At that time point urinary concentrations ranged from 4 to 20 ng/mL. The total amount of unchanged drug excreted during the first 12 hours varied between 10.3 mg and 12.3 mg corresponding to 41-55% of the administered dose. Excretion profiles for CLT are presented in figure 2.

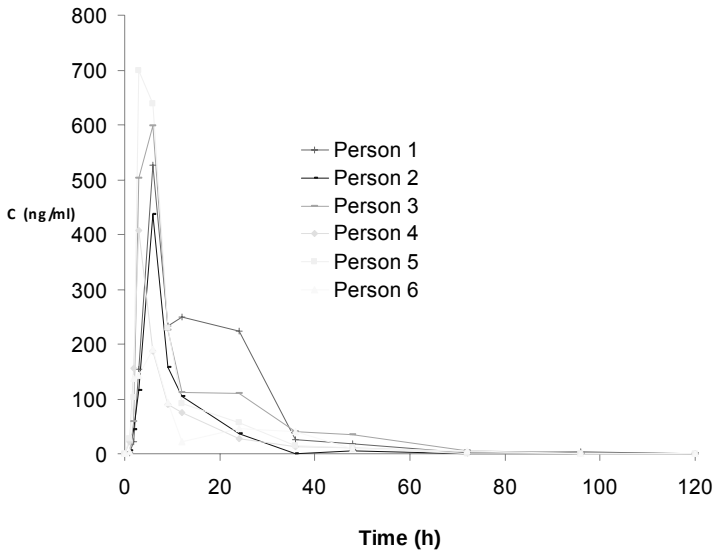

Figure 2: Urinary excretion profiles of CLT after administration of 25 mg HCT

In all 6 volunteers CLT could be detected from 1 hour after the intake. The maximum urinary concentration of CLT was reached after 3 or 6 hours and ranged between 190 and 700 ng/mL. CLT could also be detected after 120h with estimated concentrations between 0.19 ng/mL and 0.300 ng/mL. The total amount of CLT excreted corresponded to 0.5 %-2 % of the administered HCT. Peak concentrations for ACB were reached after 6 hours in all volunteers (Figure 3).

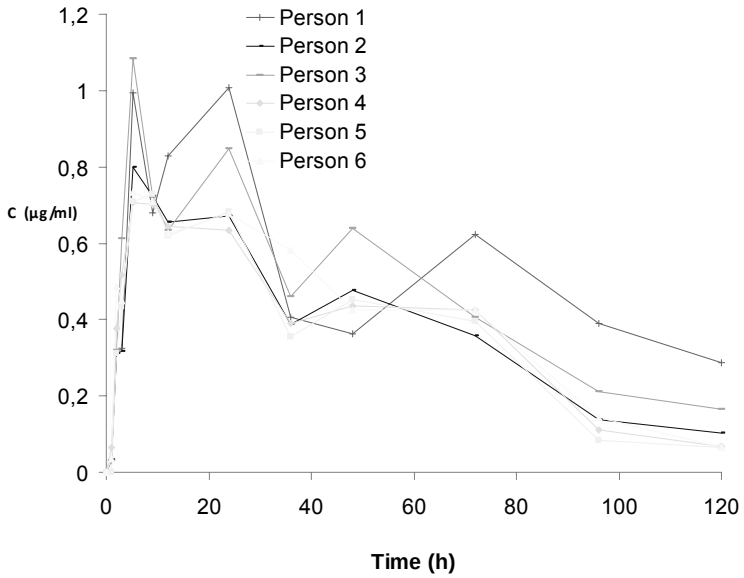

Figure 3: Urinary excretion profiles for ACB after administration of 25 mg HCT.

Maximum concentrations varied from 0.7 to 1.1 µg/mL and ACB was still present in the urine when the collection was stopped with concentrations ranging between 60 and 287 ng/mL. The total amount of ACB excreted corresponded to 3-4.5% of the administered HCT.
The purity of the administered Docspirochlor tablets was verified using the presented LC-MS/MS method. Analysis showed the presence of the parent drug HCT as well as CLT and ACB. Estimated concentrations for CLT and ACB were lower than 0.1%.

Aldactazine (15 mg altizide/ 25 mg spironolactone)

Due to the instability of the parent compound no validated excretion profiles could be established for ALT and only detection periods were determined for this compound. The detection periods for ALT were highly variable: for 2 persons ALT could be detected until 24 hours, for 2 persons 36 hours and for another two until 48 hours after administration. Excretion profiles for CLT are presented in figure 4.

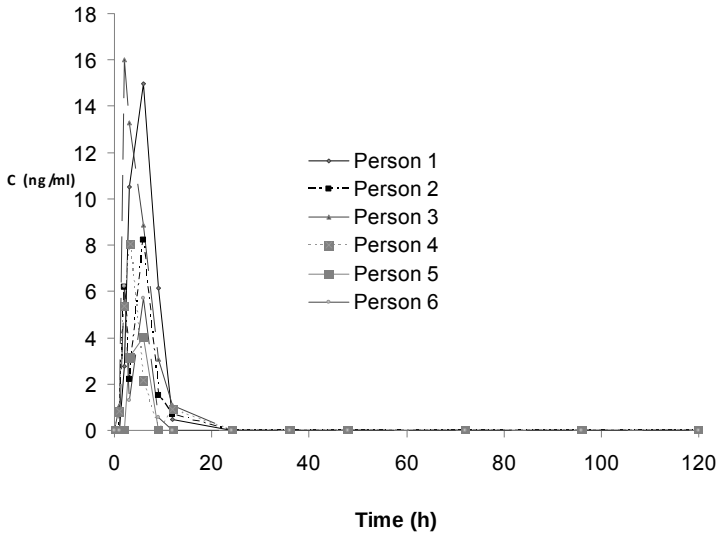

Figure 4: Urinary excretion profiles of CLT after administration of 15 mg ALT.

CLT could be detected already after 1 hour in all volunteers. The maximum urinary concentration of CLT was reached after 3-6 hours and ranged between 8.0 and 14.0 ng/mL. Detection times for CLT varied from 36 h to 120 h after administration. The total amount of CLT excreted was less than 0.1% of the administered ALT.

Besides the detection of CLT, HCT was also detected, but in concentrations never exceeding the LOQ of the method. Estimated maximum concentrations of HCT were ranging between 1 and 7 ng/mL. Detection time periods were between 48 and 120 h.
Peak concentration of ACB was already reached after 2 hours in one volunteer. For the others peak concentrations were reached after 6-9 hours (Figure 5). Maximum concentrations varied from 1.4 to 2.7 µg/mL. ACB could still be detected when the collection of urine was finished with concentrations ranging between 41 and 239 ng/mL. The total amount of ACB excreted during the first 12 h, corresponded to 11-16 % of the administered dose.

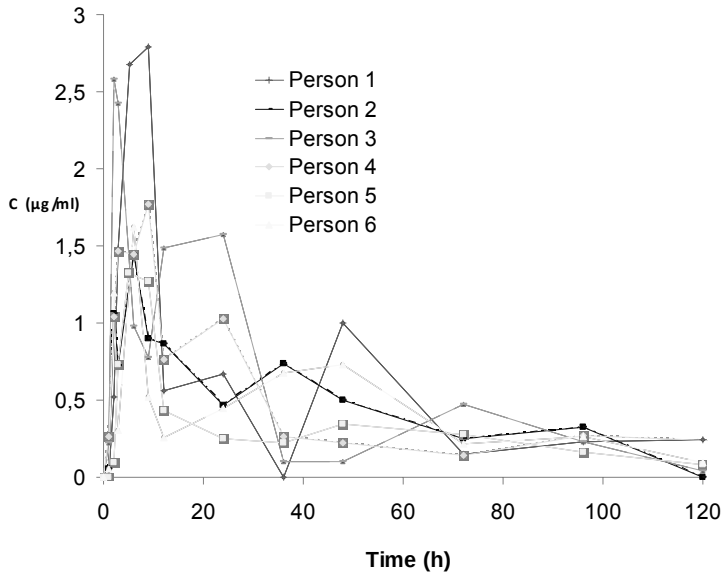

Figure 5: Urinary excretion profiles of ACB after administration of 15 mg ALT.

The purity of the administered Aldactazine pills was checked using the described LC-MS/MS method. Analysis showed the presence of the parent drug ALT but also of HCT, CLT and ACB. Estimated concentrations for HCT and CLT were below 0.1 % and lower than 2.5 % for ACB.

Conclusion

A sensitive LC-ESI/MS/MS method for the quantification of HCT, CLT and ACB in urine was developed and validated. ALT could not be validated quantitatively due to its instability and its detection was performed qualitatively only. The method was successfully applied to urine samples from two administration study.

The results of the excretion study show that after the administration of 25 mg HCT the parent substance could be detected up to 120 hour. The hydrolysis product, ACB, was also detectable until the last administration study sample at 120h. In the case of the 15 mg ALT administration, the parent drug was detected for 48 hours after intake, while ACB was detectable in the 120h sample. ACB concentrations at that timepoint were for both excretion studies fairly high and ranged from 41 to 287 ng/mL.
The long detection period and the high urinary concentrations proofs that the hydrolysis product is an ideal candidate for the long time detection of thiazide diuretics in urine.
The detection of the degradation product, ACB, as well as the presence of CLT and/or the parent substances in urine-samples can give additional evidence to support an adverse analytical finding.

Acknowledgements

The authors wish to thank WADA for financial support. Postdoctoral grants by the Flemish Ministry of Culture, Youth, Sports and Brussels (P.V.E and K.D.) and the Spanish Ministerio de Educacion y Ciencia (O.J.P.) are gratefully acknowledged. We would also like to thank Professor Dr. Thierry Gillebert and the staff of the Heart Center (UZ-Gent) for the assistance in the excretion studies. The technical assistance of Bart Waegeman was greatly appreciated.

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