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Stability study of thiazide diuretics

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Introduction

In sports diuretics are used for two main reasons: to flush previously taken prohibited substances with forced diuresis and in sports where weight classes are involved to achieve acute weight loss.
The WADA list presents several diuretics and gives 3 examples for the group of thiazides including bendroflumethiazide, chlorothiazide and hydrochlorothiazide[1]. A common property observed for thiazides is hydrolysis in aqueous media resulting in the formation of aminobenzenedisulphonamide.
This degradation product can be observed for hydrochlorothiazide (HCT), chlorothiazide (CLT) and altizide (ALT) (Figure 1). Because these thiazides have a chlorine in their structure the degradation product is called aminochlorobenzendisulphonamide (ACB) (Figure 1).
The first report related to doping describing the potential hydrolysis of thiazides was described by Thieme et al [2]. In 2002 the laboratory of Sydney highlighted the importance to include the degradation products into diuretic-screening methods [3]. The first in depth study describing the degradation of a thiazide was conducted by a pesticide laboratory describing the photodegradation of bendroflumethiazide (BFMT) at pH 3 and pH 7 [4].

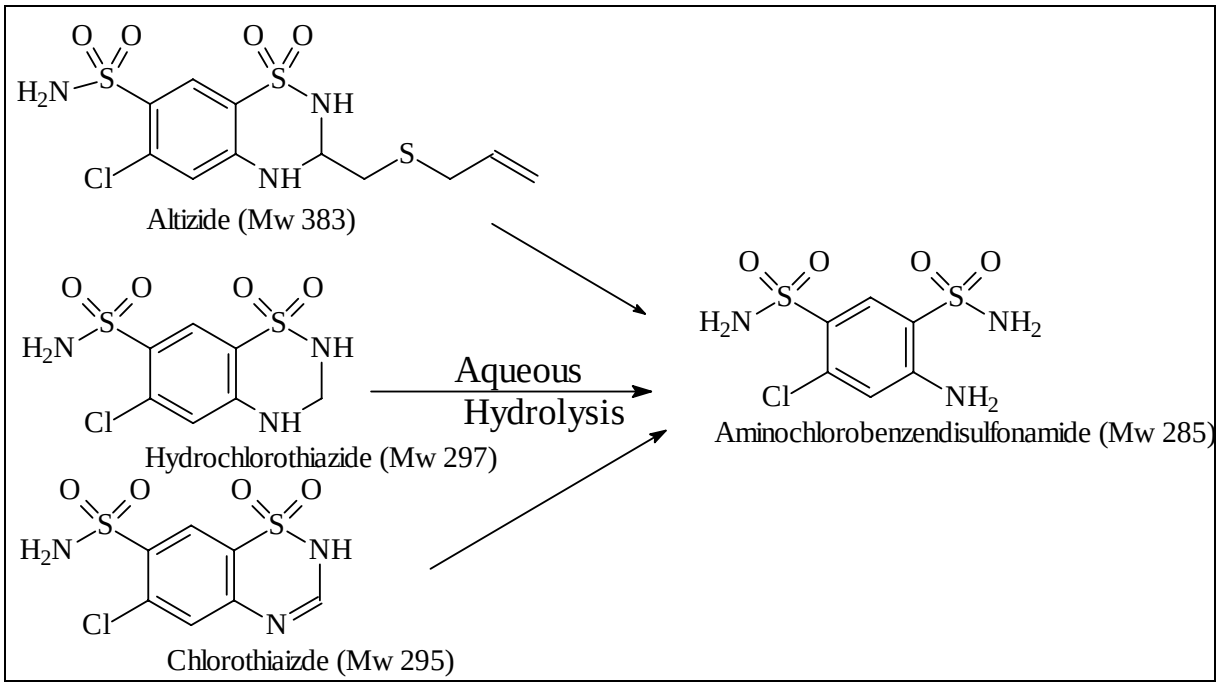

Figure 1:Hydrolysis mechanism of chlorinated thiazide diuretics.

In 2006 we investigated diuretics in the frame of a work on stability of doping agents. The outcome of this work showed that thiazides were the most unstable diuretics [5].
The degradation of the thiazides was performed on a qualitative basis without determination of degradation rates.
The aim of this study was to determine relationship of degradation, pH and temperature, both in urinary and aqueous matrices for HCT, CLT and ALT.

Materials and methods

The stability study was divided into two parts: The first part was developed to determine the formation of ACB and was based upon a qualitative approach [5]. In the second part the degradation rate was determined using a semi-quantitative approach [6].
In the qualitative part the 3 compounds were spiked in 4 buffers in triplicate at a concentration of 10 µg/mL. The solutions were then exposed to 20°C, 40°C and 60°C.
The experiments at 20°C were also performed by exposing the samples to artifical laboratory light, daylight and UV light (365nm). After finishing the experiments 50 microliter mefruside solution (20 µg/mL) were added as internal standard and the samples were cooled to 2°C using a Memmert cooling bath.

In the semi-quantitative approach degradation curves were established. In these experiments the 3 compounds were spiked in triplicate at concentrations of 10 µg/mL in both urinary, and aqueous matrices. For the experiments in water 1 mL of bidest water and 1 mL of the buffer were mixed, and for the experiments in urinary matrix 1 mL of urine and 1 mL of the buffer were mixed. The samples were evaluated at pH 5.2 and 7 (normal physiological range of urine) at 20°C , 40°C and 60°C during 5h (300 min). Aliquots were collected at 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 min. After finishing the experiments 50 µL acetazolamide (20 µg/mL) was added as internal standard.

To stop the degradation the samples were cooled to 2°C. Samples from the qualitative experiments were analysed using our routine screening method for diuretics. Samples of the semi-quantitative approach were analysed using a triple quadrupole based SRM method.

Results and discussion

Qualitative approach

Table 1: Results of qualitative degradation experiments, shielded from light, during 48 h at different pHs and temperatures.

Compound	pH	20°C	40 °C	60 °C	20°C	40 °C	60 °C
		Detection of parent			Detection of ACB		
ALT	2.0	3/3	3/3	3/3	ND	3/3	3/3
	5.2	3/3	ND	ND	3/3	3/3	3/3
	7.0	ND	ND	ND	3/3	3/3	3/3
	9.2	ND	ND	ND	3/3	3/3	3/3
HCT	2.0	3/3	3/3	3/3	ND	ND	3/3
	5.2	3/3	3/3	3/3	ND	3/3	3/3
	7.0	3/3	3/3	3/3	3/3	3/3	3/3
	9.2	3/3	3/3	ND	3/3	3/3	3/3
CLT	2.0	3/3	3/3	3/3	ND	ND	ND
	5.2	3/3	3/3	3/3	ND	ND	ND
	7.0	3/3	3/3	3/3	ND	ND	ND
	9.2	3/3	3/3	ND	3/3	3/3	3/3

ND: not detected

The results of the qualitative experiments, performed shielded from light, are presented in Table 1. At pH 2.0 all parent compounds were still present in the samples after 48 hours incubation at 20, 40 and 60°C. At this pH The degradation product ACB was observed for ALT at all 3 temperatures whereas for HCT only at 40 and 60°C. For CLT no ACB was

detected at any temperature. After incubating at pH 5.2 not all parent substances were detectable. At 40°C and 60°C, ALT could no longer be detected but ACB was present in all samples indicating the transformation of ALT into ACB. For HCT, ACB was only detected at 40 and 60°C and for CLT still no ACB could be observed. At pH 7.0 ALT was completely degraded at all temperatures. On the contrary, HCT and CLT were still present in all samples and no ACB could be detected for CLT in any samples. At pH 9.2 degradation seemed to be faster as for both HCT and CLT, ACB was detected at all temperatures. At this pH none on the parents substances could be detected at 60°C.

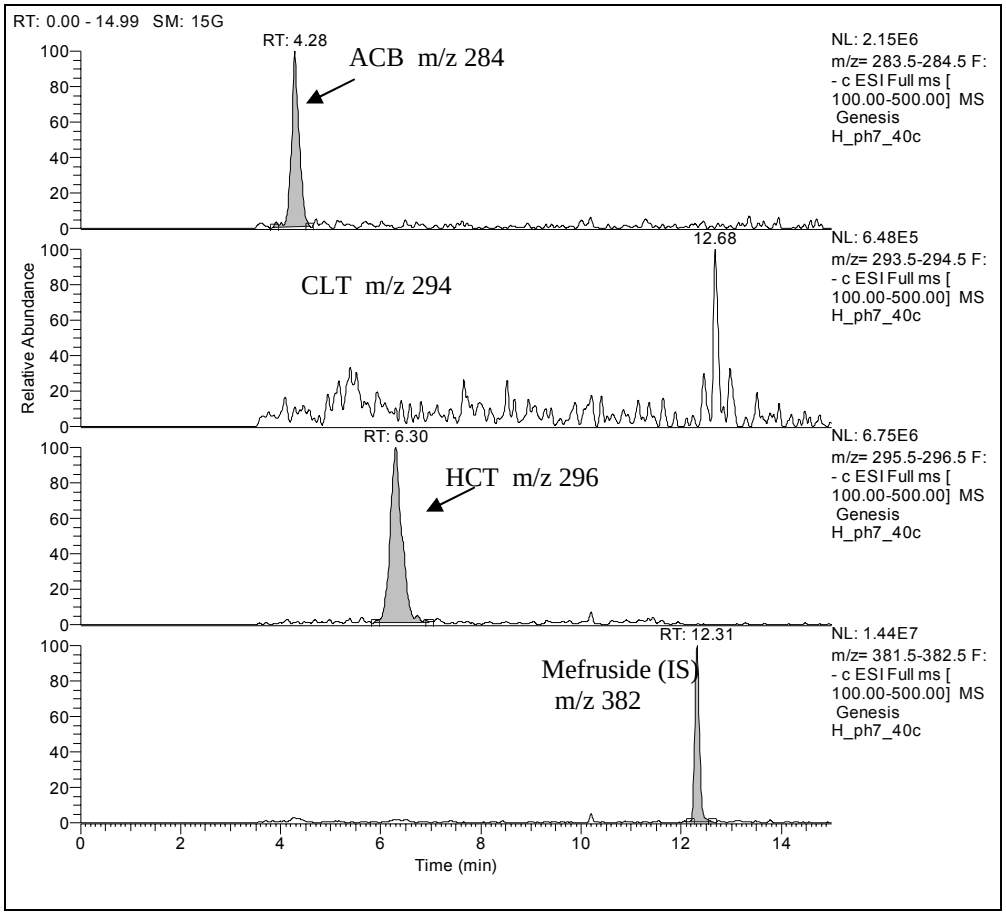

Figure 2: Extracted ion chromatograms for ACB, CLT, HCT and ALT in water, initially spiked with HCT (10 µg/mL). After exposure for 48h at 40°C and pH 7 ACB was formed (upper chromatogram). HCT was still present in the sample.

When the experiments performed at 20°C were conducted under artifical laboratory light or daylight the same results were obtained regarding the detection of the parent substances and ACB. Besides, in the samples exposed to daylight and containing ALT, a peak for CLT was detected in the samples at pH 2. At other pH values this peak was not detected. To the authors

knowledge such a transformation from ALT into CLT is not reported. For HCT the formation of CLT has been described as dehydrogenation after exposure to UVA-light [7].

Semi quantitative approach

Taking into account a previous study on the degradation of BFMT and the suggested first order mechanism of the degradation [4] linear curves were fitted through the logarithmic area ratio's ln(AR) of the target compound product ion area to I.S. product ion area versus time. In figure 3 the results for the degradation of ALT at pH 5 for different temperatures: e.g 20, 40 and 60°C is presented. The fastest degradation was observed at 60°C.

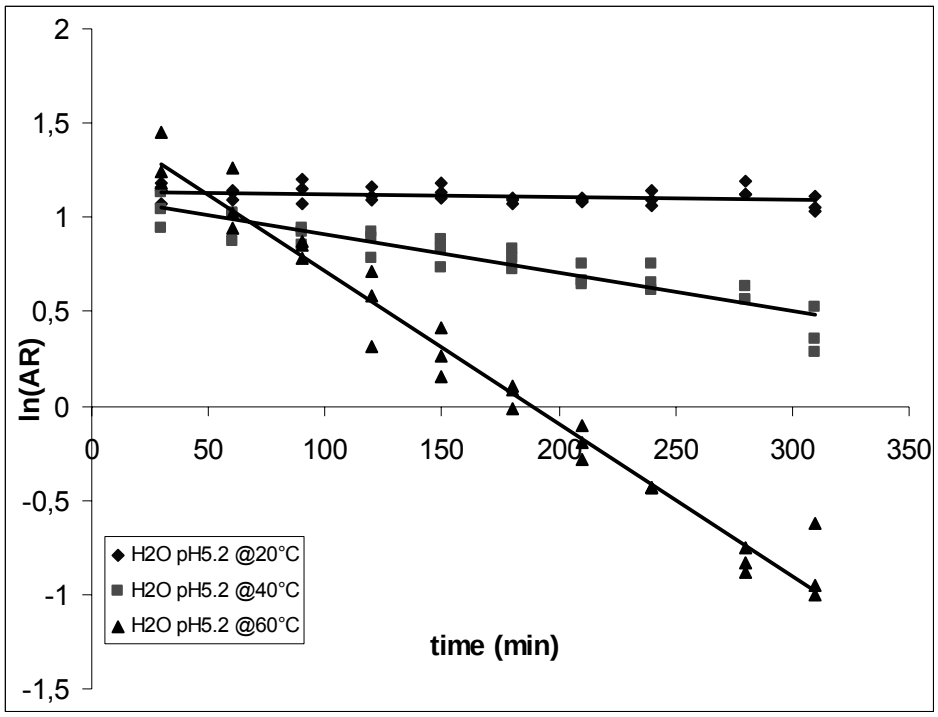

Figure 3: Degradation of altizide in water at pH 5.2.

When degradation in urinary and aqueous matrices was compared the slopes of the curves in water were significant steeper than those obtained in urine. For example, for ALT at pH 7 half of the initial amount was lost after 82 minutes whereas in water $T_{1/2}$ was determined to be 66 minutes. This faster degradation rate in water has also been observed with bendroflumethiazide [4]. A comparison between the slopes in water and urine is presented in figure 4.

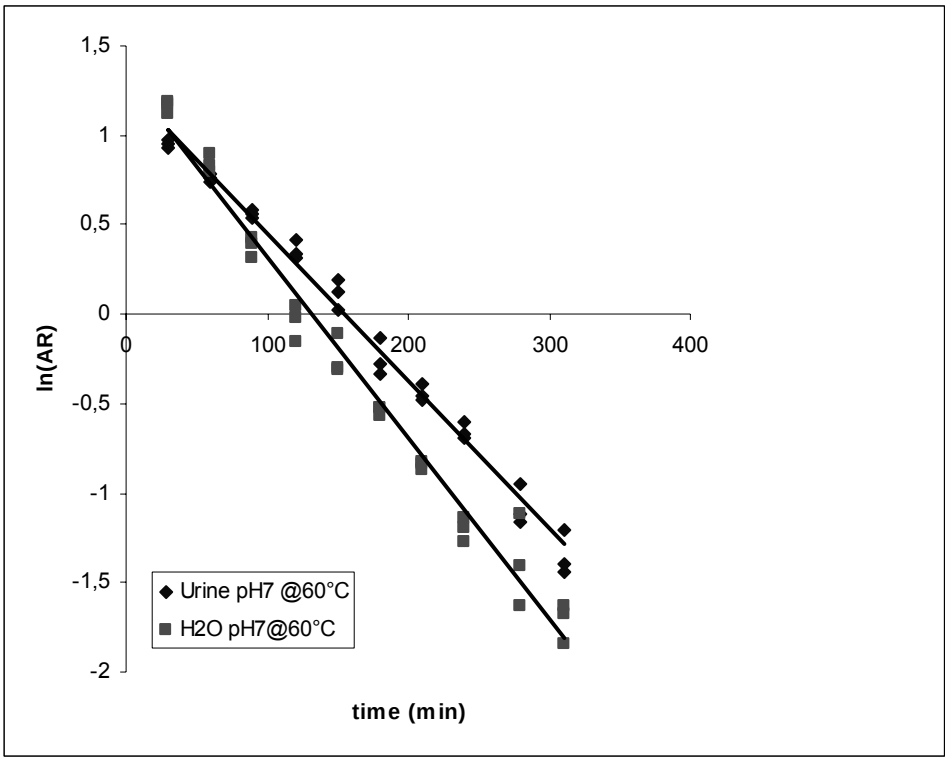

Figure 4: Comparison of degradation rate in urinary and aqueous matrices for altizide at 60°C and pH 7.

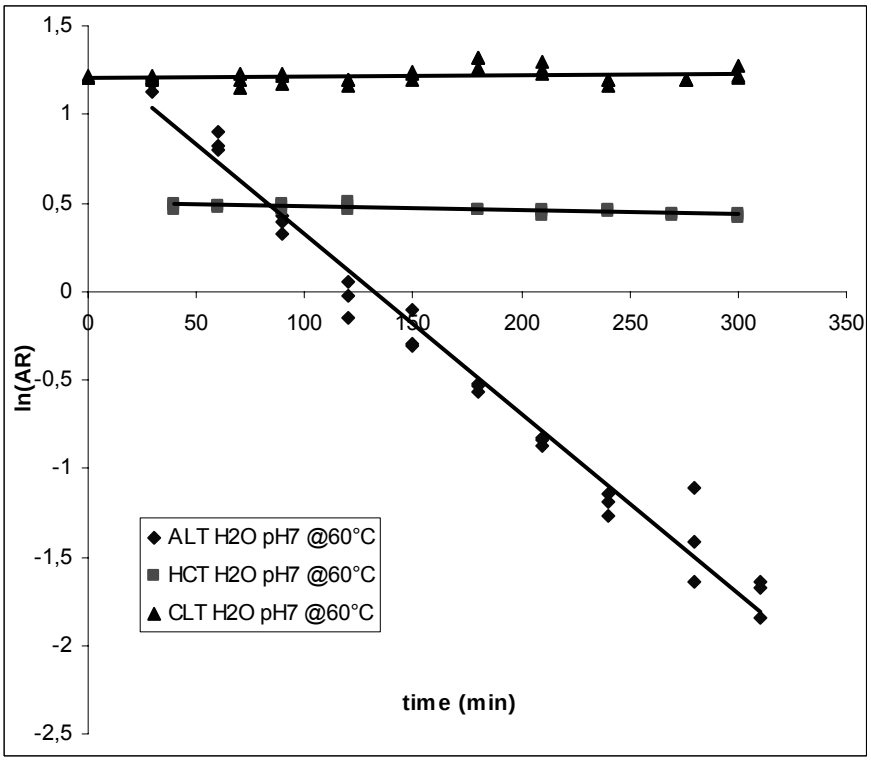

Figure 5: Comparison of the degradation for ALT, HCT and CLT at pH 7 and 60°C.

Finally, the plot of the degradation curves for the three substances at pH 7 and 60°C in figure 5 clearly showed that ALT had the highest degradation rate, CLT is the most stable and the degradation speed for HCT lies in between.

Conclusions

This work shows that thiazide drugs are subject to degradation to a different extent depending of pH, light and temperature. However not all situations have a similar effect. At pH 2, thiazide drugs were fairly stable even at elevated temperatures. The thiazide drugs degradate faster at higher pH values. Physiological pH values in urine range between 5.5 and 7.5. As can be concluded from the results, this range is critical and significant degradation can commence. Unfortunately no pH adjustment can be done to preserve thiazides in urine because other substances can be altered or degraded. Hence it is recommended to cool or freeze urine samples during transport and storage.
The substances investigated in this work exhibited different degradation rates and altizide seemed to be the most unstable one. As previously observed for bendroflumethiazide degradation in urine was slower than in aqueous media.

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