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Stability of benzodiazepines in hair after prolonged exposure to chlorinated water

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ABSTRACT

Introduction: An *in vitro* study on authentic positive samples was carried out, aiming the evaluation of the effect of chlorinated water on benzodiazepines in hair. Two subjects exposed to chlorinated water for several consecutive times were also investigated.
Method: Seven hair samples collected from autopsy cases, previously tested positive for benzodiazepines, were washed with dichloromethane and methanol. They were longitudinally divided in six aliquots of about 30 mg. An aliquot was processed without treatment while other five ones were soaked in chlorinated water (0.1% sodium dichloroisocyanurate and 0,1 M sulfuric acid at pH 5.5) for 4, 20, 24 and 30 h respectively. Hair samples were then processed following a fully validated and previously published method. Briefly hair samples were sonicated in 600 microliters methanol containing halazepam (IS) up to two hours. Ten microliters were injected in a liquid chromatographic tandem mass spectrometric (LC–MS/MS) system. Analytes were eluted from a C18 reversed-phased column. Two transitions on multiple reaction monitoring and positive ionization mode were monitored for each compound.
Result and discussions: Six compounds among benzodiazepines and metabolites were identified and quantified in the seven hair samples: diazepam (575 pg/mg), desmethyldiazepam (562 pg/mg), chlordesmethyldiazepam (173 pg/mg), desalkylflurazepam (320 pg/mg), clonazepam (three cases—195, 119 and 111 pg/mg respectively), lormetazepam (two cases—182 and 416 pg/mg respectively). Traces of 7-aminoclonazepam were identified into 2 samples. Stability of benzodiazepines in water was evaluated by soaking an aliquot of hair for up to 30 h in deionized water. No significant degradation was observed. Samples soaked in chlorinated water showed considerable decreasing from the initial concentration even after the 4-h treatment: the fastest degradation was provided by clonazepam that showed a 61% loss. The greatest loss was measured for diazepam (86% loss after 30-h soaking).
Conclusion: To the best of our knowledge this is the first *in vitro* study that evaluated benzodiazepines stability in hair after prolonged exposure to chlorinated water. The results showed that the longer the exposure the higher the degradation. Prolonged exposure to chlorinated water and sunlight must be always taken into account as possible causes of false negative results.

1. Introduction

Benzodiazepines represents one of the most widely prescribed class of drugs in the world [1,2]. Since they have fewer side effects and are much safer in overdose, they have largely substituted barbiturates as CNS-depressants drugs. However, benzodiazepines are of extreme interest in forensic toxicology, because of their effects on psychomotor function, even in recommended doses. Several deaths due to benzodiazepines overdosages, alone or in combination with other drugs, are reported in literature [1–6]. Alprazolam and diazepam are included in the list of the 10 substances most frequently detected in deaths occurred after overdose in the U.S.A. during the period 2010–2014 [1,2]. Many cases of intoxication with benzodiazepines involved children, that are more exposed to the toxic effects of this class of drugs [5–7]. The opportunity of evaluating past and/or continuative use of a drug may give valuable information about the case. So far hair represents the best biological matrix to study a past exposure to xenobiotics. Several methods for detection of benzodiazepines in hair have been developed through the years [8–13]. Some of them are extremely sensitive and are able to detect even a single drug intake [14–18]. However, a negative result should not be interpreted as an incontrovertible proof of not exposure to drugs. In fact, it is already known that cosmetic treatments, such as bleaching, could strongly affect substances concentration in hair

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[19,20]. On the contrary, the effect on the stability of xenobiotics in hair after exposure to chlorinated water it has not been yet investigated. Therefore, this study was developed to determine the effect of chlorine on benzodiazepine stability in hair via experiments with authentic samples. In addition, a case report will be discussed.

1.1. Study protocol

Two authentic samples were collected from two subjects; the first at the beginning of June and the second in September, after three months of repeated baths in a public pool. The segmental hair analysis was performed for all the samples (Table 1)

We collected seven hair samples from previous autopsy cases that previously provided positive results for benzodiazepines. We divided the samples longitudinally into 6 aliquots each, whether possible. The first aliquot was processed, following the analytical procedure described below. A second aliquot was soaked in a glass tube containing deionized water and kept at room temperature in the dark for 30 h. The other four aliquots were soaked in freshly made chlorinated water (0.1% sodium dichloroisocyanurate in deionized water, buffered at pH 5.5 with 0.1 M sulfuric acid). Time of immersion was different for each aliquot (4, 20, 24, 30 h respectively). The soaked aliquots were then taken to dryness and processed following the same analytical procedure.

Water from a public pool was collected in a plastic tube and kept in the laboratory until the complete cessation of chlorine activity (15 days). The same procedure was applied to the chlorinated water prepared in the laboratory. A positive hair sample was divided into two aliquots and eventually soaked into the two water solutions for 30 h. Eventually the two samples were processed using the method described above (Table 2).

2. Material and methods

The analytical procedure for benzodiazepines detection and quantification in hair was already fully validated and previously published [8].

2.1. Reagents

Lorazepam, alprazolam, bromazepam, flurazepam, α-hydroxyethylflurazepam, desalkyflurazepam, flunitrazepam, 7-amino-flunitrazepam, clonazepam, 7-amino-clonazepam, lormetazepam, clobazam, demoxepam, prazepam, chlordiazepoxide, midazolam, nitrazepam, zolpidem, medazepam, oxazepam, diazepam, nordazepam, α-hydroxy-triazolam, triazolam and temazepam were purchased by Lipomed (Nova Chimica, Milan, Italy), clotiazepam, estazolam, etizolam were obtained by Formenti (Formenti SPA, Milan, Italy) chlordesmethyldiazepam and brotyzolam were purchased by Ravizza (Ravizza Farmaceutici SPA, Milan, Italy), ketazolam and pinazepam were obtained by Ciba-geigy (Basel, Switzerland) and halazepam was purchased by Schering-Plough (Schering-Plough, Milan, Italy). Water was purified by filtering deionized water on a Milli-Q Simplicity 185 filtration system from Millipore (Bedford, MA, USA). Formic acid for mass spectrometry was obtained from Sigma-Aldrich (St. Louis, MI, USA). HPLC-grade methanol and acetonitrile were purchased from Mallinkrodt Baker (Milan, Italy).

The mobile phase consisted of a mixture of 0.1% (v/v) formic acid (A) and acetonitrile (B).

Table 2
Benzodiazepines detected in hair from autopsy cases and their concentrations. Samples were analyzed in a single segment (length ranging from 3 to 6 cm).

Hair sample	Benzodiazepine	Concentration (pg/mg)
Subject 1	Diazepam	575.0
	Desmethyldiazepam	562.0
Subject 2	Clonazepam	111.0
	Lormetazepam	182.0
Subject 3	Clonazepam	119.0
Subject 4	Lormetazepam	416.0
Subject 5	Desalkylflurazepam	320.0
Subject 6	Chlordesmethyldiazepam	173.0
Subject 7	Clonazepam	195.0

2.2. Instrumentation

LC–MS/MS analyses were performed with an Agilent 1100–1200 Series system (Agilent Technologies, Palo Alto, CA, USA) interfaced to a 4000 Q-TRAP (AB SCIEX, Foster City, CA, USA) with an electrospray (ESI) Turbo V™ Ion Source. The LC instrumentation was composed of a vacuum degasser, a binary pump, and an autosampler kept at 4 °C. The injector needle was externally washed with methanol prior to any injection. A kinetex core-shell C18 column (100 × 2.1 mm i.d., 5 μm particle size) (Phenomenex, Castel Maggiore, Italy) were kept at 25 °C during the analysis. Mobile phase consisted of formic acid 0.1% (A) and acetonitrile (B), and the flow rate was 0.2 mL/min. A gradient elution was developed as follows: 90% to 10% A within 5.0 min, maintaining of 10% A up to 11.0 min, and re-equilibration up to 22 min. The 32 substances monitored were then divided into 2 groups. The ESI source settings were: ion-spray voltage: +5500 V, source temperature: 350 °C, nebulization and heating gas (air): 20 and 25, respectively. Multiple Reaction Monitoring (MRM) was optimized using nitrogen as collision gas (with pressure set at level 8) and a dwell time of 30 ms. Two transitions for each substance were chosen for identification; the most intense was used for quantification purposes. Data acquisition and elaboration were performed by the Analyst® software (version 1.5.1, AB SCIEX).

Table 1
Concentrations of lormetazepam in hair of the two authentic cases.

	Hair segment collection before summer (cm)	Lormetazepam concentration (pg/mg)	Hair segment collection after summer (cm)	Lormetazepam concentration (pg/mg)
Mother			0–3 (52 mg)	<10.0
	0–2 (41 mg)	60.7	3–5 (36 mg)	<10.0
	2–4 (43 mg)	112.2	5–7 (34 mg)	11.4
	4–6 (42 mg)	144.4	7–9 (39 mg)	13.9
	6–8 (43 mg)	156.1	9–11 (35 mg)	15.2
Son			0–3 (45 mg)	<10.0
	0–2 (26 mg)	22.6	3–5 (34 mg)	<10.0
	2–3.5 (30 mg)	31.8	5–7 (34 mg)	<10.0
	3.5–6 (34 mg)	16.0	7–9 (28 mg)	<10.0