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Bromantan: - Japanese Experience –
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in
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BROMANTANE: - Japanese experience -

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INTRODUCTION

Several steroid test samples have been invalidated since 1994 by the IOC laboratories in Montreal, Lausanne, Huddinge and Tokyo because of the presence of the unknown undeclared agent. The identification of this bromine containing compounds was made by Ayotte in the middle of 1996 in cooperation with International Amateur Athletic Federation. The compound, bromantane, is a adamantane derivative that has the para-bromaniline side chain, and was developed in Russia as immuno-stimulator. In Russia, it has been used among militaries and sportsmen as the unauthorized medicine. The later in centennial Olympic in Atlanta, nine bromantane cases were found but the findings were not resulted in disqualification because of the legal problem. This paper refers to a positive case of bromantane. Our results demonstrated that over dose of bromantane could manipulate the urinary steroid profiles. It is not just an interfering and a co-elution but also the manipulation of the chromatographic behavior of the steroids. The simple extraction procedure to eliminate the influence of bromantane on the steroid profiles is given.

MATERIALS AND METHODS

The sample preparation and GC/MS analysis for the normal steroid test were done by our published procedures. The procedure consists of XAD-2 micro-column extraction, methanol elution, separation of free and conjugated steroids, deconjugation with Glufatase (Arylsulfatase/β-glucuronidase from Ampullaria), diethylether extraction of azyrene and following trimethyl silylation with enolization. GC/MS was performed by both HP5970 MSD (unit mass resolution) and JMS-700 MStation from JEOL (high resolution up to 60,000).
RESULTS AND DISCUSSION

Positive case of bromantane:

Our first positive case was found at the international indoor track and field meet in Gunnma, Japan on February 1996. The male athlete have declared the recent medication on form as follows:

V-B complex, Pentrexyl (anti-biotic), Multi vitamin, Creatine phosphate, Carbohydrate, Xylocaine (local injection)

Bromantane use was not declared by the athlete. The pH and the density of the specimen on arrival to the laboratory were 5.3 and 1.040 respectively. The urine specimen had a strong dark yellow color, and the methanol elute of the steroid extract had a yellow-green fluorescence. The viscosity of the specimen was too high.

The results of the initial steroid test have shown the elevated T/ET values (T/ET>10), but the quality of the MS chromatogram was poor. (figure-1, figure-2) Androsterone (A), etiocholanolone (E), dihydrotestosterone (DHT) were not completely separated from bromantane and its hydroxylated metabolites. Therefore, the sample was followed by the further modified analytical procedures.

Modification-1: Diethylether extraction, reduced injection volume

The injection volume of the derivative was reduced to 0.5 μ l (1/4). Identification of the steroids by GC retention times became possible. However, Hydroxy bromantane-O-TMS was still seen on the MS chromatogram and the shape of the peaks was relatively poor.

Modification-2: Cation exchanger (AG 50W x 8)

Urine sample was taken through the Dowex AG50W x 8 column (H+ form) under slightly acidic medium (pH=5.3), and the void fraction was collected in stead of XAD-2 column extraction. Some yellow interfering compounds were held by the column but the interfering compounds were still seen on MS chromatogram. The GC retention time of the interested compounds were much delayed.

Modification-3: Cation exchanger, reduced injection volume

The extract from the modification-2 was used but only 0.5 μ l (1/4) of the derivative was injected onto GC column. GC retention times of the steroids were identical but the slight delay was observed.
Modification-4: n-Pentane extraction under neutral pH

n-Pentane was used as the extraction solvent instead of diethylether. The addition of potassium carbonate was omitted in order to reduce the recovery of the basic compounds. Bromantane metabolites were eliminated using less polar organic solvent. However, the retention time of the interested compounds was slightly delayed.

Modification-5: n-Pentane extraction under neutral pH, reduced injection volume.

In this method, 0.5 μl of the extract from modification-4 was injected onto GC column. Bromantane and the metabolites were successfully removed, and GC retention time was identical. This modification was the best suitable for the determination of the urinary steroid profiles.

Modification-6: TEAP LH20 (Anion exchange)

Triethyl hydroxypropyl sephadex LH-20 was synthesized according to the procedure of Axelson et.al.. 3) The free and the sulfated steroid fraction was removed, and glucuronide fraction of the neutral steroids was collected. The yellow elution band of bromantane positive sample was eluted much more rapidly than other normal urine samples. Some part of the interference was co-eluted with steroid glucuronide. Thus, the elimination could not successfully be performed.

The typical MS chromatograms are shown in figure-3. Each extracts were analyzed both by quadrupole MS with MS resolution 500 and by high resolution MS with MS resolution 7,000. High resolution instrument gave the better analytical results but its use was not enough because of the GC retention time shift. The elimination of bromantane and its metabolites from the extract was not easy so that the best strategy was to reduce the extraction recovery of bromantane and its metabolites at the first extraction step in the analysis. Overfeeding of the GC column is appeared more easily when considered the concentration of the steroids in the sample. It is expected from our studies that bromantane metabolites can interact with steroids (e.g. π-π interaction) or can mask the inner surface of GC column when they are present in the extract in large amount. This is provably due to the special chemical property of bromantane and its metabolites. (figure-4).
CONCLUSION

This study represented the masking effect of bromantane in the steroid screening. Overdose of bromantane can confuse the interpretation of the analytical results. The interference is successfully eliminated by the extraction with n-pentane without the addition of potassium carbonate. Thus, the steroid profiles are unmasked.

REFERENCES


Figure-1 MS chromatogram of the testosterone test of bromantane positive urine. (Ion trace m/z: 432)
Figure 2. MS chromatogram and MS spectra of bromantane metabolites.
Figure 3 Differences of chromatographic behavior due to the sample preparation procedures. (Bromantane positive)
Figure-4 3-D structure of bromantane