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Determination of aminorex in human urine samples by GC–MS after use of levamisole

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ABSTRACT

The Drug Enforcement Administration (DEA) reports that as of October 2010, 79% of all cocaine seized in the United States contained levamisole. The equine conversion of levamisole to aminorex has been demonstrated. However, the metabolic fate of levamisole in humans is unknown. Nevertheless, as aminorex is amphetamine-like and hallucinogenic, it may be used as an adulterant to increase the effects of cocaine. We report here the results of *in vivo* studies demonstrating for the first time that not only equine, but also canine and human metabolism all result in aminorex formation. Levamisole and aminorex were extracted from real urine samples by liquid–liquid extraction and identified and quantified by GC–MS (identification by 3 ions per substance, LLOQ at 0.15 ng/ml for both).

1. Introduction

Levamisole made its first appearance as a cocaine adulterant in 2004 [1]. Three years later Italian authorities seized 28 kg of cocaine hydrochloride found to contain nearly 10% levamisole [2]. A 2009 report from the Netherlands found only the commonly used adulterants – phenacetin, hydroxyzine and diltiazem. However, the authors of that same report also observed that cardiac and hallucinatory effects in that region's cocaine abusers were, for no apparent reason, being reported more frequently. They suggested that the adverse effects might have been due to the adulterants. One way to account for the apparent increase would have been the presence of levamisole, although this was not apparent at the time. These speculations were followed five years later by controlled studies demonstrating the equine conversion of levamisole to aminorex [3,4] (Fig. 1). The metabolic fate of levamisole in humans has never been established. We report here the results of our studies, which definitely prove that not only equine, but also canine and human metabolism of levamisole all result in the production of aminorex, are all similar. Detection and quantification of levamisole and aminorex were carried out by means of a liquid–liquid extraction at pH 9 and GC–MS detection, adapted from routine amphetamines procedure.

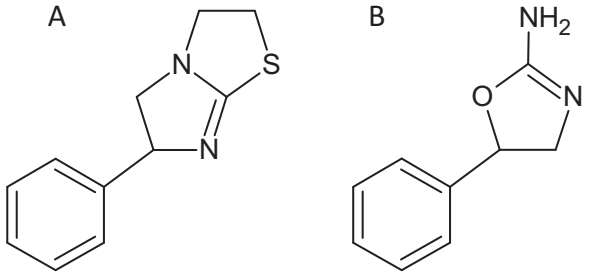

Fig. 1. Structure of levamisole (A) and aminorex (B).

2. Materials and methods

2.1. Reagents

Aminorex and levamisole phosphate were obtained from Cerilliant (Round Rock, TX), mephentermine (N,2-dimethyl-1-phenylpropan-2-amine), used as internal standard, was acquired from Chemical Research (Rome, Italy), N,O-bis (trimetylsilyl) trifluoroacetamide (BSTFA) was purchased from Sigma (S. Louis, MO). All other reagents were obtained from J.T. Baker (Deventer, Holland). Blanks, calibrators and control were prepared in certified drug-free urine purchased from Polymed (Firenze, Italy).

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Table 1
Validation data: average concentration, accuracy and inter-day precision ($n = 6$).

Levamisole					
Nominal concentration (ng/ml)	20	40	60	80	100
Average concentration (ng/ml)	19.58	39.03	59.56	78.48	89.15
Accuracy (%)	2.87	3.12	5.01	7.54	9.06
Inter-day precision (%)	8.56	5.67	7.25	3.34	7.12
Aminorex					
Nominal concentration (ng/ml)	20	40	60	80	100
Average concentration (ng/ml)	18.78	37.56	56.87	76.53	97.47
Accuracy (%)	2.15	4.02	6.13	7.32	9.01
Inter-day precision (%)	6.42	5.67	7.55	4.05	8.47

2.2. Real samples

Urine samples were collected from 8 healthy volunteers (4 males weighting about 70–80 kg, 4 females weighting about 55–65 kg, all subjects from 35 to 45 years old), and from 4 dogs each weighting approximately 20 kg. All the dogs were being treated with levamisole because of worm infestation. Urine specimens were collected from the human volunteers immediately before, and then 3 and 6 h after the administration of 47 mg and 58 mg of levamisole by oral route (47 mg for female and 58 mg for male). Samples were taken from the dogs immediately before and 3 and 6 h after the administration of 120 mg of levamisole by subcutaneous injection.

2.3. Samples treatment and analysis

All urine samples were initially screened by enzyme multiplied immunoassay technique (EMIT, Siemens Viva-E, Siemens Healthcare Diagnostics, Camberley, UK) for cocaine metabolite (cut-off = 0.3 mg/l) and amphetamines (cut-off = 0.5 mg/l).

For confirmatory analysis levamisole and aminorex were extracted from urine samples (1 ml + 10 ng mephentermine as internal standard) with 5 + 5 ml of diethylether after stabilization at pH 9. Diethylether was evaporated under nitrogen stream. Both reduced volume samples and trimethylsilyl derivatives, obtained by adding 50 μl of N-Methyl-N-(trimethylsilyl) trifluoroacetamide at 75 °C for 15 min, were injected in a gas chromatography-mass spectrometry (GC–MS) apparatus (Agilent Technologies with Inert MSD: 7890A, 5975C, 7693 autosampler, with a phenylmethylsilicone 5% capillary column, 30 m × 0.250 mm × 0.25 μm). The injector was set at 300 °C and splitless injection was performed; the column oven temperature was programmed at 80 °C for 1 min, then increased to 300 °C at 20 °C/min for 3 min. Identification was performed monitoring five ions for each analyte: m/z 73, 101, 148, 203, 204 for levamisole, m/z 56, 91, 118, 145, and 162 for aminorex, m/z 73, 91, 162, 291, and 306 for bis-trimethylsilyl aminorex, and

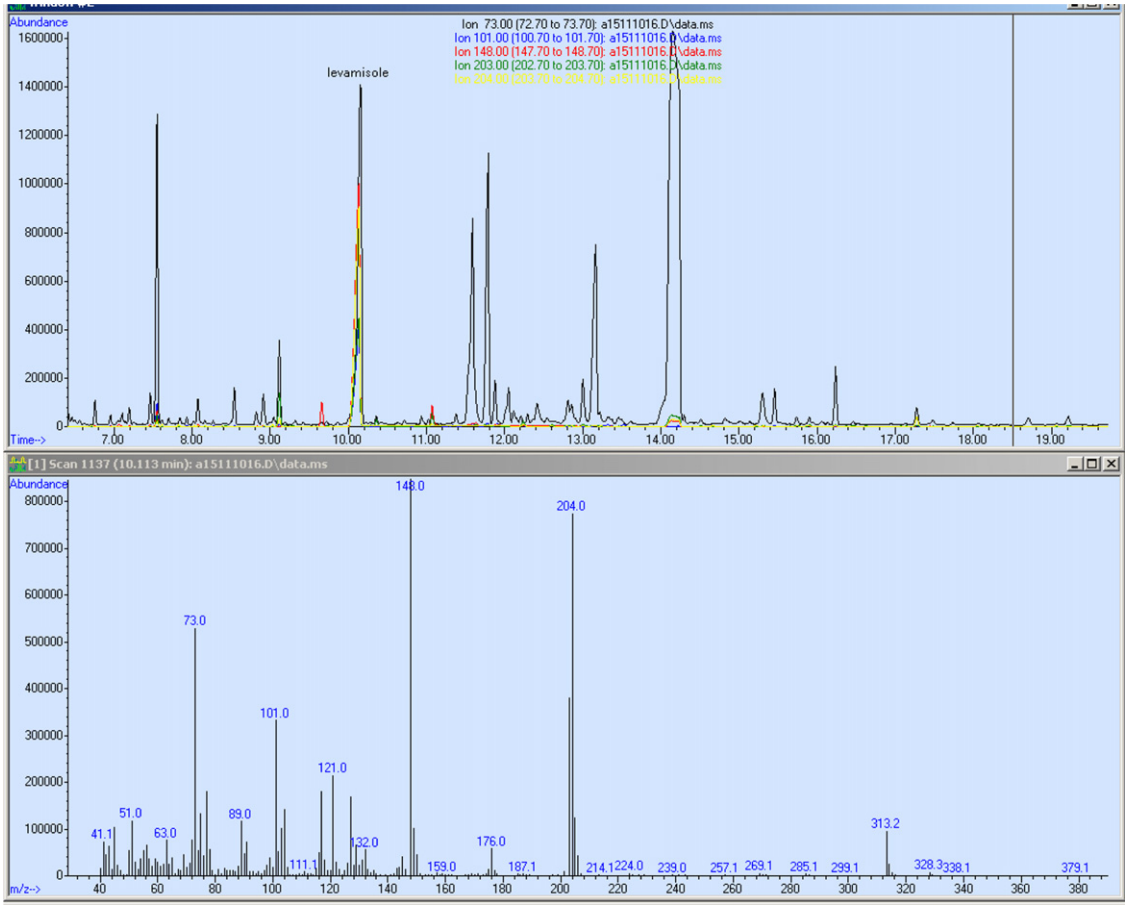

Fig. 2. Extracted ion chromatogram of a positive urine sample (A), levamisole mass spectra (B).

Table 2
Concentration of aminorex and levamisole in humans (ng/ml urine).

Sample (1–8)	3 h		6 h	
	Levamisole	Aminorex	Levamisole	Aminorex
Mean	40.63 (30.05–53.22)	30.63 (22.52–38.12)	20.63 (12.45–28.34)	28.32 (20.08–43.6)
Standard deviation	8.62	5.48	6.56	7.95

m/*z* 72, 148, and 91 for mephentermine. The samples were analyzed also in scan mode at the same chromatographic conditions.

2.4. Quantitative analysis of levamisole and aminorex and method validation

Quantification was performed by construction of 6-point calibration curves prepared by spiking drug-free urine at the following final concentrations: 0, 20, 40, 60, 80 and 100 ng/ml. Blank and positive controls were analyzed within each batch run. Inter-day precision and accuracy were calculated as relative standard deviation and bias on positive control specimens ran on six different days (Table 1). Quality control (QC) samples (comprising a negative urine sample that had been fortified with 50 and 25 ng/ml of levamisole and aminorex) were analysed in duplicate for each batch of samples in order to verify that the analysis was in control.

3. Results

All urine samples collected before levamisole administration tested negative at EMIT, while the specimens collected after levamisole administration tested very close to the cut-off value for amphetamines (results between 428 and 440, cut-off: 500 ng/ml).

No interfering substances were observed in any of the samples collected from dogs and volunteers before levamisole administration and then analyzed by liquid–liquid extraction and GC–MS. Initially, both levamisole and aminorex were qualitatively identified in all canine and human samples collected at both 3 and 6 h after levamisole administration. Identification was based on the acquisition of five ions per compound, on the comparison of scan spectra with references and libraries [3–7] and corroborated by the identification of the bis-trimethylsilyl derivative of aminorex in the derivatized samples.

Acceptable linear regression was obtained for both calibration curves (levamisole: $y = 0.134x + 0.034$, $R^2 = 0.997$; aminorex: $y = 0.202 + 0.125$; $R^2 = 0.995$). Inter-day precision and accuracy were always better than 10% for levamisole and 12% for aminorex (Table 1). The lower limit of quantification (LLOQ) was determined to be 0.15 ng/ml for both analytes considering that precision and accuracy were satisfying at that concentration. The limit of detection (LOD) was set at the same concentration.

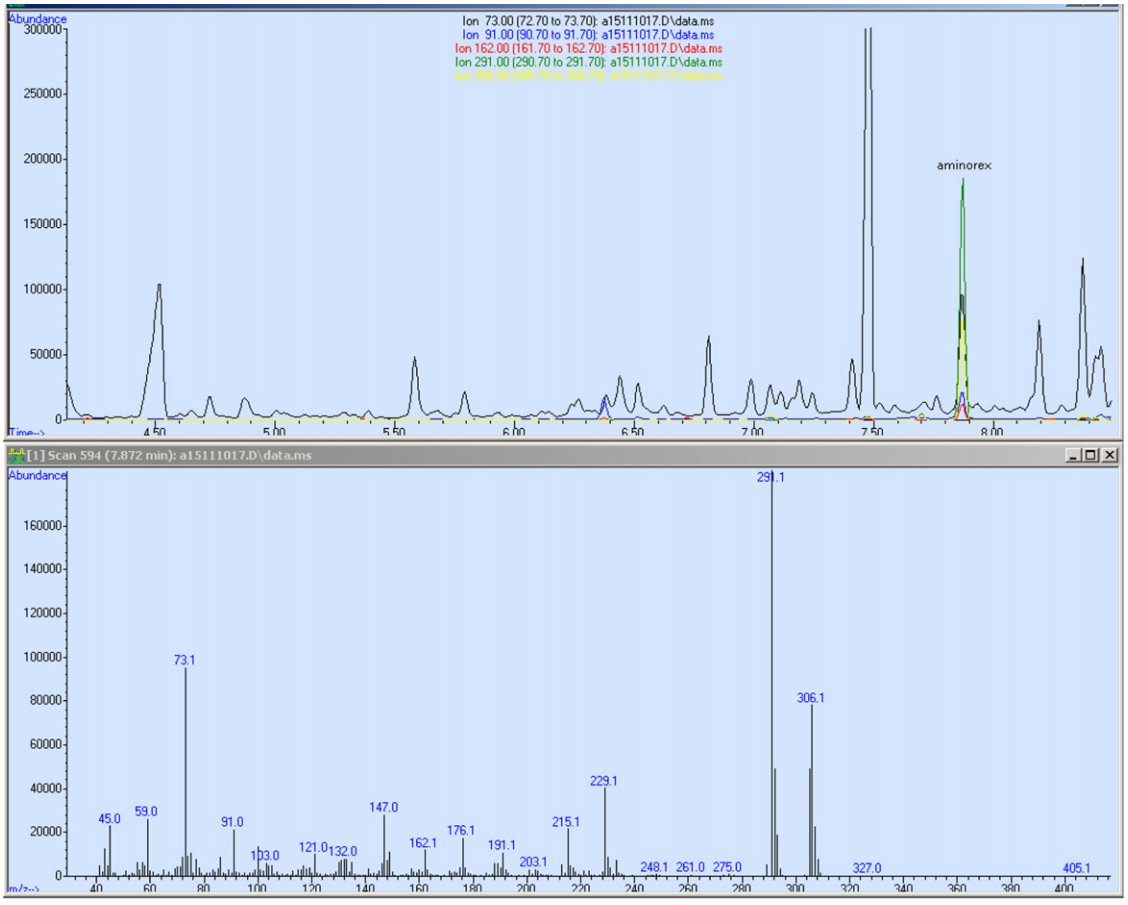

Fig. 3. Extracted ion chromatogram of a positive urine sample (A), aminorex mass spectra (B).

Results obtained from individual urine specimens are summarized in Table 2, including mean concentration, standard deviation, and median for each group.

Twelve stored human urine samples previously found to be positive for cocaine and benzoylecgonine at GC–MS confirmation by the method routinely used in the laboratory, were anonymized and analyzed for levamisole and aminorex. Four out of 12 urinary samples tested positive to both levamisole and aminorex when analyzed by the method described above (Figs. 2 and 3).

4. Discussion

In 1991, the U.S. Food and Drug Administration (FDA) approved levamisole (under the brand name of Ergamisol®) for use as adjuvant therapy with fluorouracil for the treatment of colorectal cancer. In spite of the limited FDA approval, levamisole was used "off label" as an immunomodulator in the treatment of numerous, unrelated disorders. Except for use as an anthelmintic, where it is extremely effective, results when used with other disorders were unimpressive [8,9]. It was withdrawn from the U.S. market in 1999 because a clear association with agranulocytosis had become apparent [10]. It remains available as a veterinary medicine in the United States, Italy, Canada, and South America.

Data from the DEA's Signature Program (a federal program that tracks the composition of cocaine seizures within the United States) showed less than 1% of the samples tested in 2001 contained levamisole. By July 2009, that number had risen to approximately 69 per percent [11]. In Florence, Italy, from November 2009 to November 2010, 34% of cocaine seizures analyzed in our laboratory were found to contain levamisole. Similar results have been observed elsewhere in Italy [7], and the rest of Europe [12], though the true percentage is not known.

Levamisole now permeates the U.S. cocaine supply [8,10,13–15], suggesting that two disorders should become increasingly common: agranulocytosis and necrotizing vasculitis. Neither condition is very common and their diagnosis, particularly in a young person, should initiate a search for levamisole-contaminated cocaine.

Clusters of cocaine–levamisole related agranulocytosis were reported in the U.S. at the end of 2009 [11] and in the spring of 2010 [10]. Levamisole-contaminated cocaine seems to result in a distinctive clinical syndrome, including the presence of circulating plasmacytoid lymphocytes, increased bone marrow plasma cells, with mild megakaryocytic hyperplasia. More than half of those who become ill will have antineutrophil and HLA-B27+ antibodies [10]. Vasculitis induced by levamisole has been recognized for more than a quarter of a century and also appears to be the result of circulating immune complexes [16].

The United States government maintains a Patient Registry for Primary Pulmonary Hypertension (PPH Registry) that tracks PPH patients from 32 medical centers throughout the United States [17], but the registry excludes all PPH patients suffering from other disorders, including drug abuse. By definition it would not be sensitive to any sudden aminorex-related increases in PPH. Complicating the issue further is that all of the research on aminorex and PPH was performed more than 40 years ago, and very little is known about the subject, although the clinical presentation is fairly clear: rapidly progressing exertional dyspnea is the first and dominating symptom in all cases, and most users became symptomatic in less than one year [18].

5. Conclusions

For the first time aminorex has been identified as levamisole metabolite in human (and canine) urine, confirming human *in vivo* conversion of levamisole to aminorex. In addition, we found that four out of 12 forensic urine samples from cocaine users were positive for both levamisole and aminorex. This means either that the other eight samples had not been adulterated or, alternatively, it could mean that one of the enzymes responsible for the conversion of levamisole to aminorex is polymorphic.

According to the European Monitoring Centre on Drugs and Drug Addiction (EMCADA) 3.3% of all European adults used cocaine in 2009 and that 2.5 million individuals were lifetime users. The results of our study show that many, are unwitting aminorex users as well [19]. The most recent U.S. government figures date from 2008, at which time there were an estimated 1.9 million current cocaine users aged 12 years or older, comprising 0.7 of the total U.S. population [20]. This poses a grave public health menace to the developed world, not only for the occurrence of levamisole-induced agranulocytosis, but also for a potential epidemic of pulmonary hypertension in chronic cocaine abusers, which may well begin sometime in 2011.

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