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Analytica Chimica Acta 586 (2007) 426–431
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Ex vivo formation of gastric metabolites of clenbuterol: Preliminary characterisation of their chemical structure

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Received 28 June 2006; accepted 24 July 2006
Available online 29 July 2006

Abstract

The epidemiology of clenbuterol food-borne intoxication outbreaks indicates a possible discrepancy between the severity and long duration of clinical symptoms, and the presumed dose ingested as parent compound residue. In this work, we explore the possibility that clenbuterol could undergo to a biological transformation, in presence of salivary nitrites, at gastric pH (<3). Human salivary specimens were drawn before and after meal, accounting for the different physiological nitrite content (40 and 400 μmol L^{-1}, respectively, as average). Clenbuterol (10 μmol L^{-1}) was then incubated within the pH range 2–6 and possible products monitored by liquid chromatography–mass spectrometry (LC–MS), drawing at regular intervals serial aliquots of the incubation mixture. With respect to controls, two differential peaks were noted along with a quantitative bio-transformation of the parent compound, at pH values ≤3. Under pre-meal conditions, a 4 mono-nitro compound was identified as main metabolite, whereas under post-meal condition a second metabolite, showing a complete de-chlorination, along with the probable presence of three nitro groups on the aromatic ring, was revealed. The reaction was highly reproducible and the kinetics suggested the involvement of nitrogen-related free radicals. The results are discussed in the light of the possible formation of pharmacological active tissue-bound residues as cause of symptoms severity.

Keywords: Clenbuterol; Saliva; Nitrites; Bio-transformation

1. Introduction

Clenbuterol, a beta 2 adrenergic agonists licensed as anti-asthmatic in human and veterinary medicine in the European Union only, has progressively found a worldwide illegal use as growth promoting agent, due to its capability to elicit lypolisis and to promote muscle hypertrophy through stimulation of the corresponding tissues receptors, thus leading to the production of lean meat, particularly appreciated by the consumers [1].

Several collective food-borne intoxication outbreaks have been reported since early 1990 where clenbuterol was identified as causative agent. The most recent reports occurred in Portugal [2], Hong Kong (Republic of China) [3] and Mexico [4].

Accounting for a 100 g of edible tissue ingestion, often residues concentrations were in the range 0.5–5 ng g^{-1} (total drug intake about 0.05–0.5 μg/toto). Although in some cases clenbuterol residues in the meat or liver were higher, yet such dietary intake often did not appear to be high enough to justify the severity and the long duration (>48 h) of the clinical symptoms [5,6], if we consider that a therapeutic dose (30 μg per person, three times a day) is still considered safe [7].

Previous studies on clenbuterol pharmacokinetics in man [8] and rat [9] describe most of the drug assumed in a single dose by oral route is excreted within 48 h, and urinary metabolites appears to retain a negligible pharmacological activity [10].

Owing to the above, the attention was addressed to other extra-hepatic drug bio-transformation pathways of clenbuterol,

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0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.07.061

that could partially justify such clinical findings. Within this frame, the possibility that such drug could undergo to nitrosation in the stomach in presence of salivary nitrite was considered, due to the presence of a primary aryl-amine and a secondary alkyl-amine in its structure [11]. Such gastric nitrosation has been already described for propranolol [12] and other beta adrenergic antagonists drugs [13] as possible consequence of their therapeutical administration by oral route. In this work, we describe the possible gastric nitrosation of clenbuterol, incubating *ex vivo* human salivary specimens drawn pre- and post-main meals (nitrite concentration ranging from 40 to 400 μmol L^{-1}, respectively) with the drug at 10 μmol L^{-1}, a concentration well representative of the incurred residues levels that caused collective intoxications.

2. Experimental

Salivary specimens (HS) from 10 health volunteers were drawn and pooled together, according to pre- and post-meal drawing time, respectively.

Clenbuterol as hydrochloride salt, Griess reagents for the colorimetric NO_2^- determination were purchased from Sigma–Aldrich Italia (Italy), sodium nitrite from Merck (Germany). Methanol, acetic acid, hydrochloridic acids were of highest purity grade from Carlo Erba (Italy). Cut-off filters (M_w 10.000) were purchased from Millipore, Italia (Italy). GLP 22 pHmeter (pH 0–12) (Crison, Italy) spectrophotomer from Beckmann Coulter, chromatographic column: X Terra C-18 150 × 2.1 mm (5 μm) (Waters), LC-MSD SL 1100 series from Agilent Technologies, supplied by a Whatmann 72–75 nitrogen generator.

To obtain HS with different NO_2^- contents, HS was collected from healthy volunteers, after overnight fasting, before or 1 h after meal. Before collecting HS, the mouth was washed three times with water. The collection of HS was initiated 1 h after meal and continued for 1 h. HS was maintained at 0 °C during collection and was centrifuged at 12,000 × *g* for 10 min,

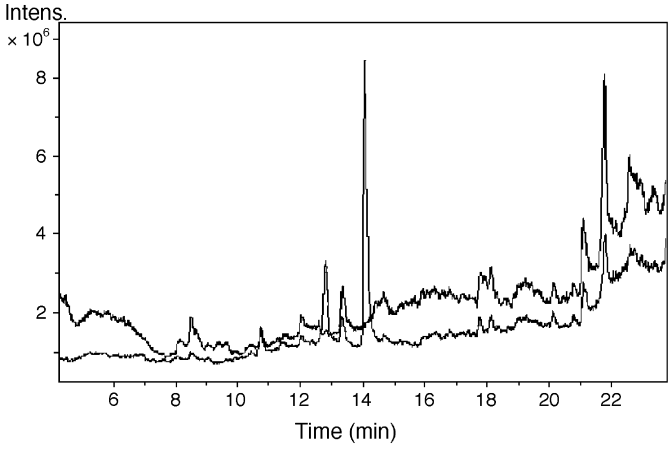

Fig. 1. Bio-transformation of clenbuterol (RT = 12.8 min) under *ex vivo* pre-prandial condition along with the formation of M1 product (RT = 14.2 min), incubation time 30 min, pH 2: blank HS as control.

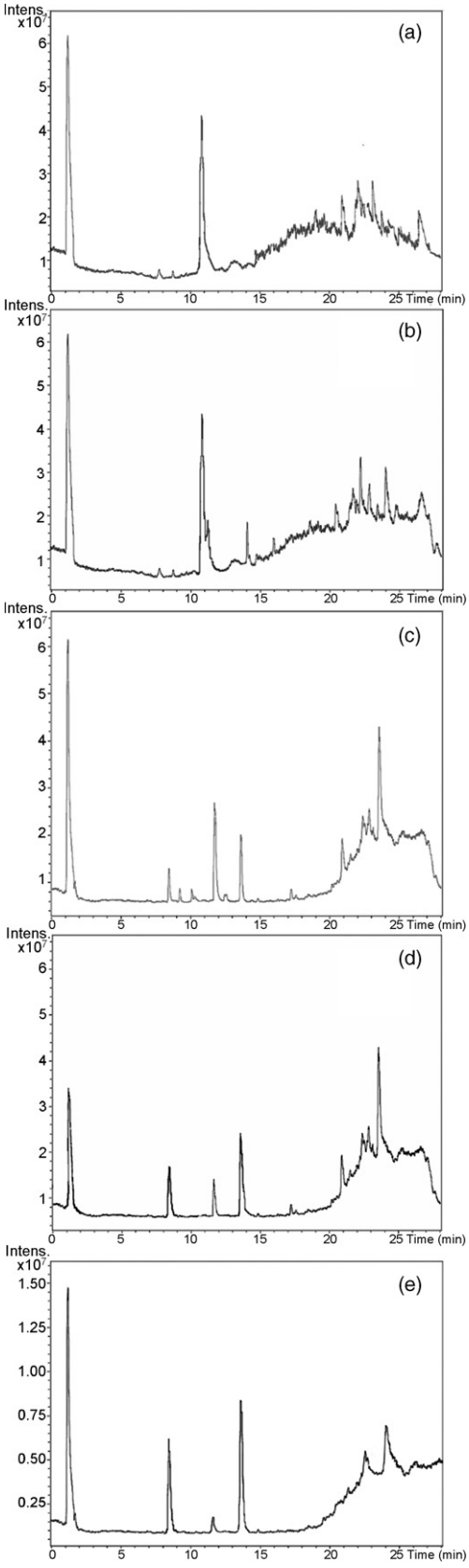

Fig. 2. Bio-transformation of clenbuterol (RT = 11.8 min) under *ex vivo* post-prandial condition at pH 2, after: (a) 1, (b) 2, (c) 3, (d) 4 and (e) 5 min of incubation, along with the formation of M1 (RT = 13.8 min) and M2 (RT = 8.6 min) products.