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Detection of beta-blockers in human urine by GC-MS-MS-EI: perspectives for the antidoping control

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Abstract

We have developed a general method for the detection of beta-blockers and/or of their metabolites in human urine. The method comprises a pretreatment procedure (enzymatic hydrolysis, liquid/liquid extraction and derivatization by pentafluoropropionic anhydride, PFPA), carried out on an initial aliquot of 2.5–5.0 ml of urine, and the instrumental analysis of the derivatives, performed by GC-MS-MS (ion trap) with electronic impact ionization (EI). The GC-MS-MS analysis allows to isolate and to characterize specific fragments of the original molecular structure, and particularly the fragments originating from parent ion clusters specific for all beta blocking drugs, giving rise to $m/z = 366$ and 202 ions respectively. MS-MS analysis of the parent ion allows checking for the presence of the above-mentioned peaks in the GC-MS chromatogram. The proposed method is capable of detecting a great variety of known (and possibly also of newly synthesized) beta-blockers, with an average sensitivity limit of 20 ng/ml of drug/metabolite in urine. The method is presently being evaluated as a general screening protocol to be followed by an antidoping laboratory to detect illicit beta-blockers administration to the athletes.

1. Introduction

Beta-adrenergic receptors antagonists (beta blockers, BB) (see Fig. 1 for molecular structures) are a class of drugs widely used in clinical pharmacology, for the treatment of cardiovascular diseases (hypertension, ischemic heart disease, certain arrhytmias), and of glaucoma, for the prophylaxis of migraine, and for controlling acute panic symptoms in anxiety-provoking situations [1,4–8]. As for other receptor-active agents, pharmacological properties of BB depend on both receptors localization in the various tissues and the activity of the corresponding sympathetic nerves; this explains why BB does not affect heart function at rest but are very effective during exercise and/or stress, i.e. when sympathetic control of the heart is dominant.

In the illicit pharmacological support to sport competition, BB are used to reduce the cardiac frequency and to minimize tremors, in order to improve the performance in skill-based sport disciplines [9,10].

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The most widely used techniques for the trace analysis, in the human urine, of residues of BB (unchanged drugs and/or their metabolites) comprise a pre-treatment procedure (enzymatic hydrolysis, liquid/liquid extraction, and derivatization) and the subsequent analysis by gas-chromatography-mass spectrometry in selected ion monitoring (GC-MS-SIM) [11–16]. Such a procedure is roughly the same, with minor variations in terms of both the experimental and instrumental conditions, for all different International Olympic Committee (IOC) accredited laboratories.

The major drawback of these methods is due to the fact that the knowledge of the molecular structure of the searched drugs/metabolites and the availability of the corresponding reference standards is required: the consequence is that the detection of any 'novel' BB is not possible by these methods.

We are presenting a GC-MS-MS method that is in principle capable of identifying the portion of the molecular structure common to all drugs acting as blockers of the beta adrenergic receptors:

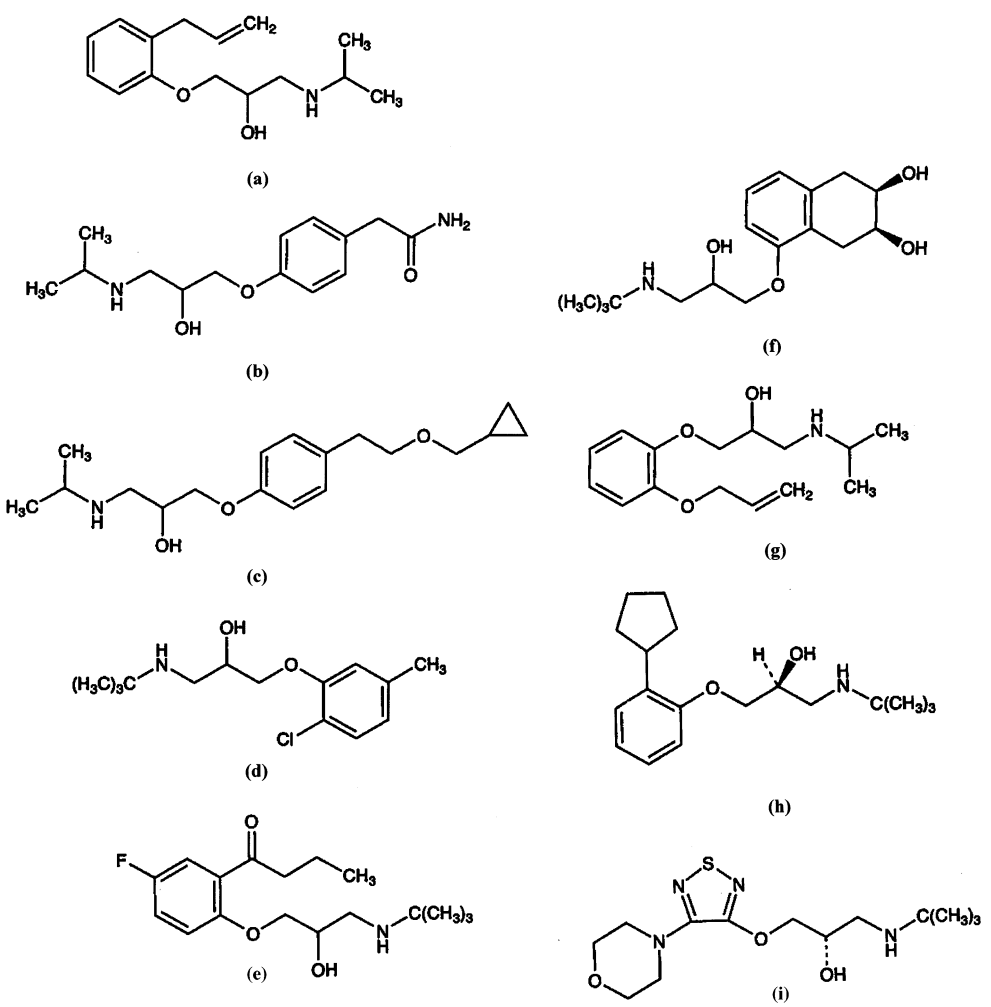

Fig. 1. Molecular structures of all the antagonists of the beta-adrenergic receptors considered in this study. (a): alprenolol; (b): atenolol; (c): betaxolol; (d): bupranolol; (e): butofilolol; (f): nadolol; (g): oxprenolol; (h): penbutolol; (i): timolol. The residue -[O–CH_2–CHOH–NH–$C(CH_3)_2$R] (where R=H or -CH_3), recurrent in all beta-blockers, is the portion of the molecule selected for the MS-MS analysis.

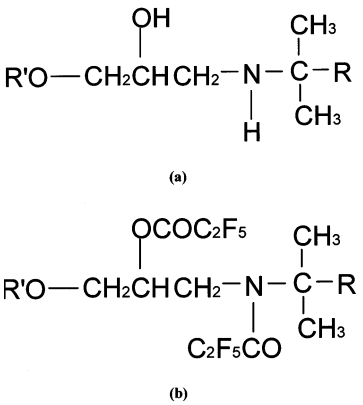

Fig. 2. Molecular structure of the recurrent portion of a generic beta-blocker before (a) and after (b) derivatization by PFPA, R=H or CH_3.

such a method could allow the detection of any beta-blocking agents, including those drugs specifically developed and/or chemically modified and employed to cheat the antidoping test.

The proposed method comprises the pre-treatment of the urine samples, structured as follows

1. Enzymatic hydrolysis of few (2.5–5.0) ml of urine, followed by pH correction; liquid/liquid extraction with organic phase (Et_2O:*t*-BuOH 10:1);
2. Pre-concentration and derivatization by pentafluoropropionic anhydride (PFPA); and
3. The subsequent instrumental analysis by GC-MS-MS-EI-SIM, carried out according to two different GC temperature programs and two different MS methods.

2. Experimental

2.1. Instrumental apparatus and reagents

All GC-MS-MS-EI assays were performed on a ThermoQuest GCQ Ion Trap MS-MS system (ThermoQuest Italia S.p.A., Rodano MI, Italy); the reference GC-MS-NCI assays were performed on a Hewlett Packard 5973 GC-MS-NCI system (Hewlett Packard Italia S.p.A., Cernusco sul Naviglio MI, Italy).

All standards, reagents and the enzyme β-glucuronidase (*E. coli*) were supplied by Sigma Chemical Co., St Louis (MO, USA).

All solvents were analytical grade. Positive reference urines were obtained by excretion studies performed on volunteers.

2.2. Urine pretreatment

Urine samples were pre-treated according to the following procedure:

1. Enzymatic hydrolysis
   - 1.1. 2.5–10 ml of urine;
   - 1.2. Brought to pH 5.2 with acetate buffer;
   - 1.3. Added with 20 μl β-glucuronidase;
   - 1.4. Kept at 50°C for 3 h;
   - 1.5. Correction of pH to 9.5 with solid carbonate buffer, addition of 3 g NaCl.
2. Liquid–liquid extraction
   - 2.1. Liquid/liquid extraction by 10 ml of Et_2O:*t*-BuOH (10:1);
   - 2.2. Centrifugation, (3500 rpm for 5 min);
3. Pre-concentration and derivatization
   - 3.1. Concentration of extract to 1–2 ml (N_2 flow).
   - 3.2. Addition of internal standard (3-OH-4-methoxy-phenethylamine).
   - 3.3. N_2 flow to dryness.
   - 3.4. Derivatization by PFPA (50 μl in ciclohexane) at 70°C for at least 40 min.

The general structure of the underivatized and derivatized drug is given in Fig. 2.

2.3. GC conditions

Carrier gas: He; column: HP1 (cross linked methyl siloxane, 18 m); injector: $T = 240$°C, constant flow rate = 0.6 ml/min, injection type: splitless.

Chromatographic assays were carried out according to two different temperature programs:

Temperature program # 1 (more rapid, useful as a general screening method): 130°C 1 min, 6°C/min to 240°C, then 25°C/min to 300°C.

Temperature program # 2 (more selective, useful as a pre-confirmation method): 100°C 3 min, 8°C/min to 235°C, then 30°C/min to 300°C.

2.4. MS conditions

2.4.1. Ion trap parameters

Method #1 (parent 366): precursor ion: m/z 366, width 1.5, time 10 ms; excitation volts 0.75, q 0.225, time 15 ms; product ions: 202 and 366.

Method #2 (parent 406–408): precursor ion: m/z 407, width 2.5, time 8 ms; excitation volts 0.75, q 0.225, time 15 ms; product ions: 202, 366 and 406–408.

2.4.2. NCI SIM parameters

All conditions are the same, with slight modifications, of the screening procedure presently followed by the antidoping laboratory of Rome, based on the GC-MS-NCI-SIM analysis of pentafluoropropionic (PFP) derivatives [10].

3. Results

Fig. 3 shows characteristic GC-MS-MS chromatograms obtained by a typical screening assay (GC conditions: temperature program #1). Plots refer to a blank urine (a) and to two BB, representative of the main groups considered in this study: alprenolol (R = H) (b) and penbutolol, (R = CH_3) (c). Results obtained by MS method #1 (parent 366, above) and by MS method #2 (parent 406–408, below) are also compared.

As it can be seen, MS method #1 (parent 366) presents a sensitivity limit (which is roughly the same for all BB, independently of the nature of the R group) of the same order of magnitude of the reference technique; while MS method #2 (parent 406–408) ensures a significant improvement in sensitivity when R=H, but at the same time a drastically reduced sensitivity when R=CH_3. MS method #2 also allows a preliminary identification of BB subclass according to the nature of the identified fragment (R=H and R=CH_3 giving rise to peaks 408 and 406 respectively).

Fig. 4 shows the chromatographic plots (GC conditions: temperature program #2) of all BB considered in this study, grouped in lots of three: alprenolol, butofilolol and timolol (Fig. 4a); oxprenolol, atenolol and nadolol (Fig. 4b); bupranolol, penbutolol, betaxolol (Fig. 4c).

Fig. 5 shows a comparison between the lowest detection limit of the screening method here proposed (GC temperature program #1 and both MS methods) (a) with the reference GC-MS-NCI technique (b). Data refer to a reference atenolol spiked solution (20 ng/ml in blank urine).

4. Discussion

In the last two decades a wide variety of new compounds endowed with activating or inhibiting activity on beta adrenergic receptors has been designed, synthesized, tested and commercialized. In many instances, the exhaustive pharmaco-toxicological characterization of newly synthesized molecules has not been completely carried out, especially as far as the comparison of efficacy, receptor selectivity and adverse effects is concerned. However, the multiple sites of actions of this class of drugs suggest a considerable interindividual variability in terms of the desired pharmacological effects, leading to the diffusion of an increasing number of different BB.

For an antidoping laboratory, this situation imposes the development and application of a screening method capable of detecting all (more or less common, more or less diffused) BB, with no risk of false negatives.

From a general point of view, structural differences at the level of the R′ group (see again Fig. 2) sharply affect the main physico-chemical and pharmacological properties of the drug. For indeed, all basic pharmacological parameters characterizing the different BB considered in the present study vary in a very broad range of values: so, for instance, the oral bioavailability vary from 10% (alprenolol) to 90% (penbutolol); the range of plasma halflife (in h) is comprised between 1 and 3 (oxprenolol) and 20–23 (penbutolol); while the volumes of distribution (l kg^{-1}) vary from 1.2 (oxprenolol) to 10.4 (penbutolol) [1–4].

Despite these differences, the residue -[CH_2–CHOH–CH_2–NH–$C(CH_3)_2$R] (with R=H or -CH_3) is recurrent for all BB synthesized so far; at the same time, it is specific for this class of drugs [17,18].

We have therefore developed a GC-MS-MS method based on the identification of ion fragments specifically derived from this peculiar portion of the molecule. Experiments performed on reference standards, on spiked urine samples and on real urine samples (negative and positive), allowed to obtain the following results:

1. Both the GC temperature programs allow the detection of BB in human urine, program # 1 being more suitable as a general screening method, especially whenever a high analytical capacity is required, and a great number of samples have to be processed in short times; while program # 2, being more selective, allowing also a preliminary identification of the specific BB subclass.

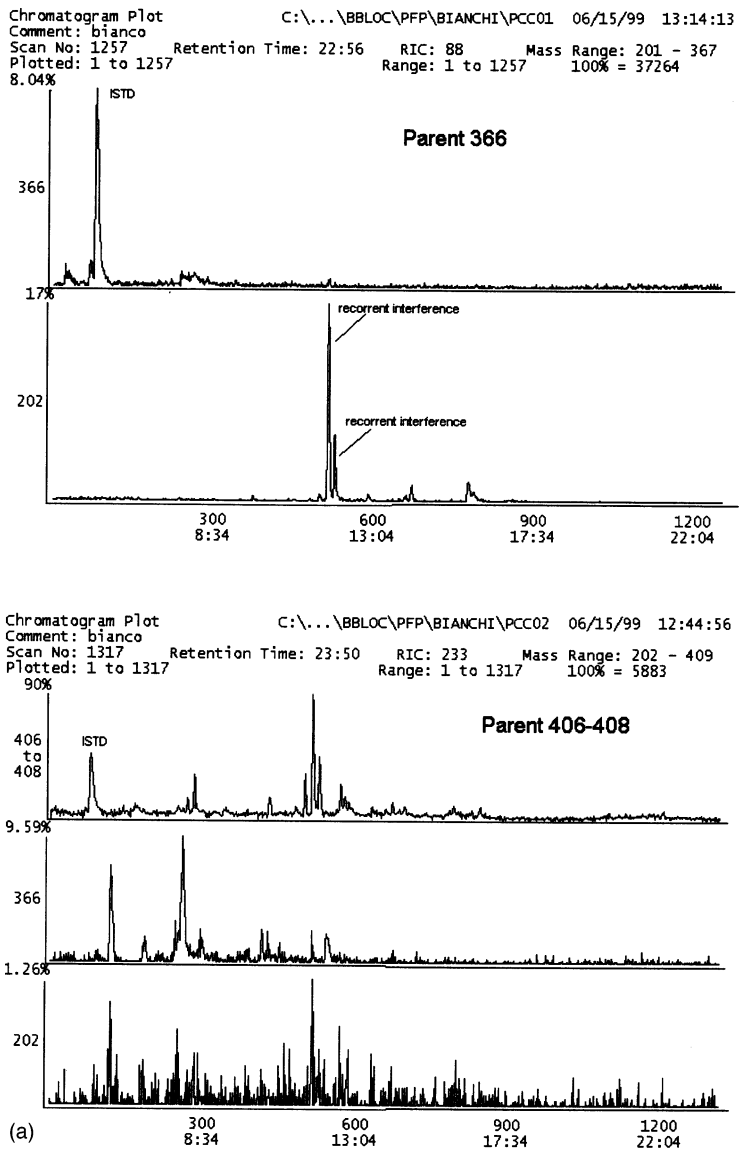

Fig. 3. GC-MS-MS-EI chromatograms obtained by GC temperature program # 2 (screening method) and both MS methods # 1 (parent 366, above) and # 2 (parent 406–408, below). (a): blank urine; (b): alprenolol; (c): penbutolol.

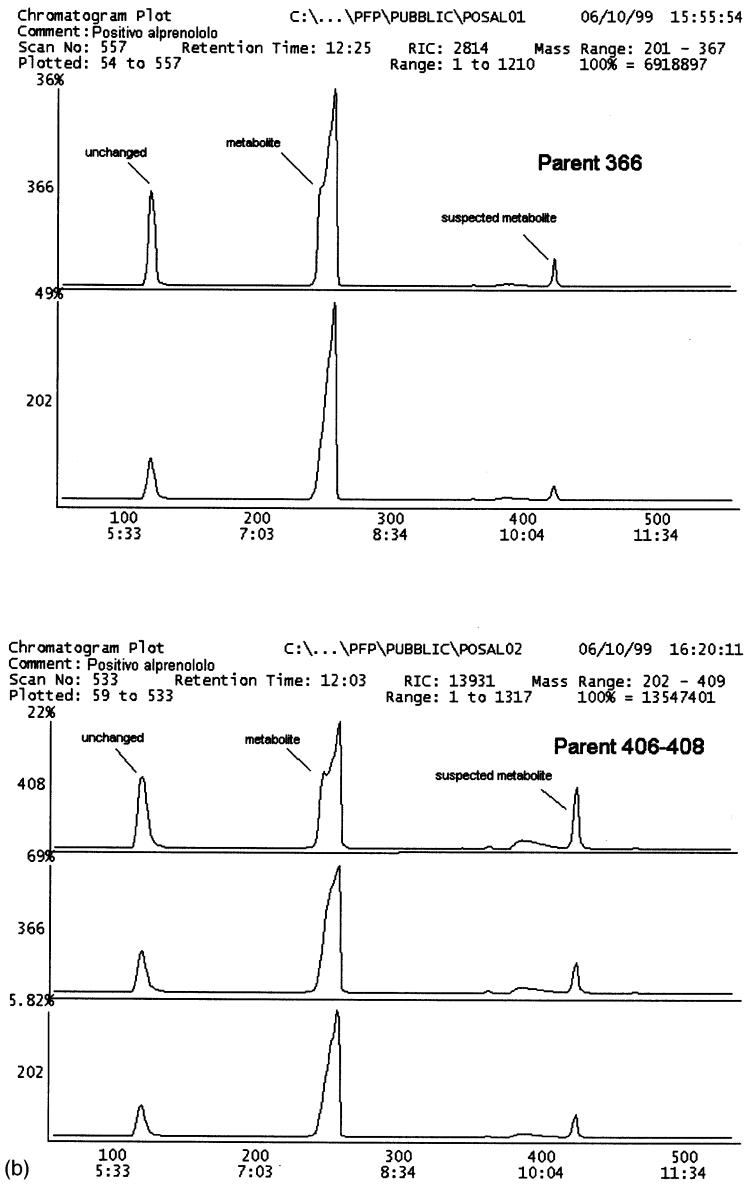

Fig. 3. (*Continued*)

2. All samples positive for BB gave rise to chromatograms with one or more peaks in the parent ion clusters m/z 406 or 408 (depending on the specific subclass) as well as m/z 366.
3. All chromatograms respective to blank samples never show any peak corresponding to both fragments $m/z = 366$ and 202;
4. All positive samples present additional peaks, usually of reduced intensity, not detected by other reference techniques: these peaks could be due to either the presence of other metabolites and/or to degradation products formed during the storage of the urine.
5. The sensitivity of the technique depends on both the specific molecular structure of each

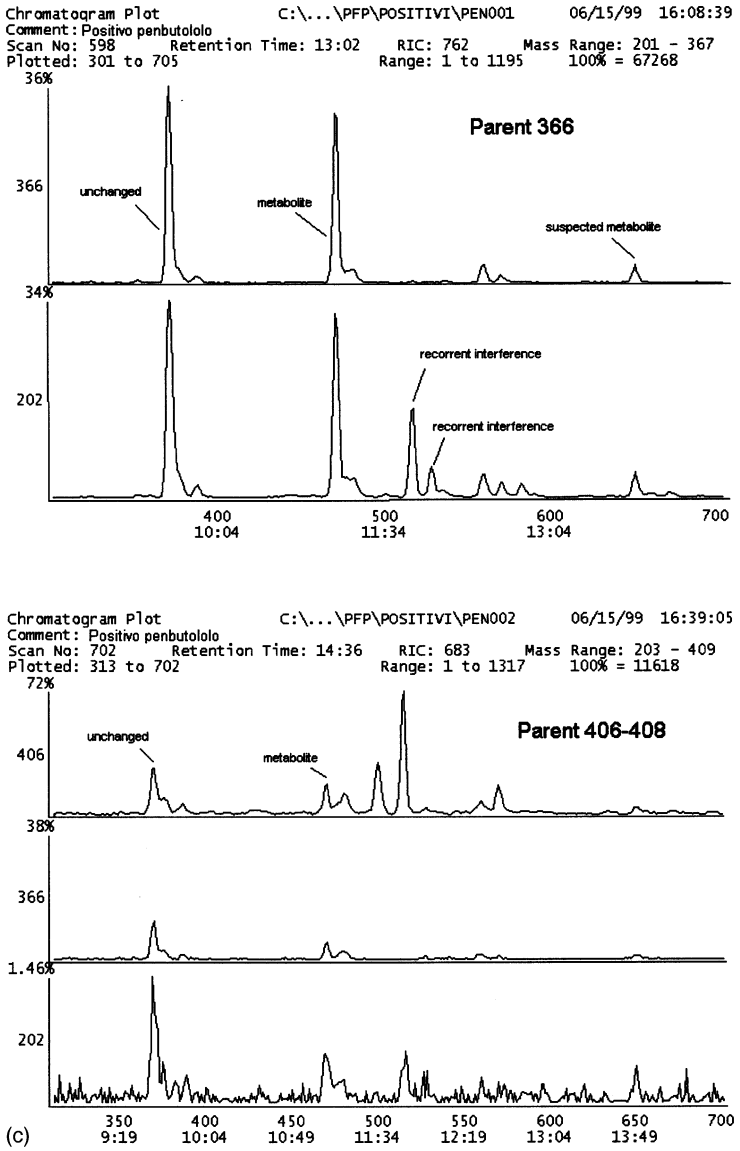

Fig. 3. (*Continued*)

compound and the ion trap acquisition method, but it is generally comparable to that of the reference GC-MS-NCI-SIM technique.

5. Conclusions and future studies

The method here presented seems to satisfy all the general requirements of an effective antidoping analytical screening protocol, and the additional demands imposed by the peculiar class of doping agents here considered. The method appears very promising for the screening analysis of athletes urine in the antidoping laboratory, also in view of the possibility of performing a wide screening of all common and uncommon BB (in

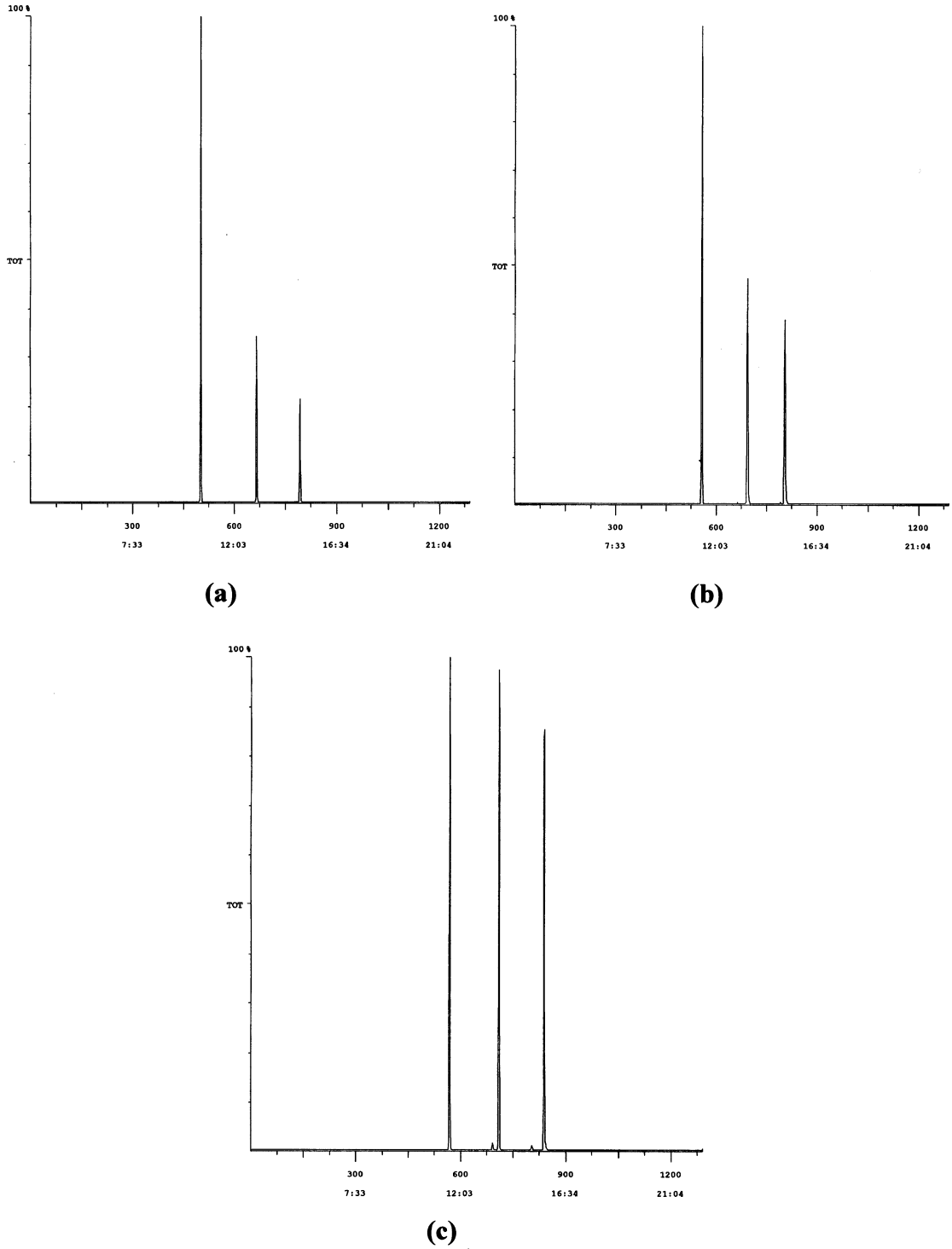

Fig. 4. GC-MS-MS-EI chromatograms of the nine considered beta-blockers (three lots of three compounds), obtained by GC temperature program #2 (which allows better separation among peaks) and MS method #1 (parent 366). Retention times are given in parenthesis: (a): alprenolol (10′34″), butofilolol (13′03″), timolol (14′57″); (b): oxprenolol (11′25″), atenolol (13′27″), nadolol (15′07″); (c): bupranolol (11′35″), penbutolol (13′42″), betaxolol (15′39″).

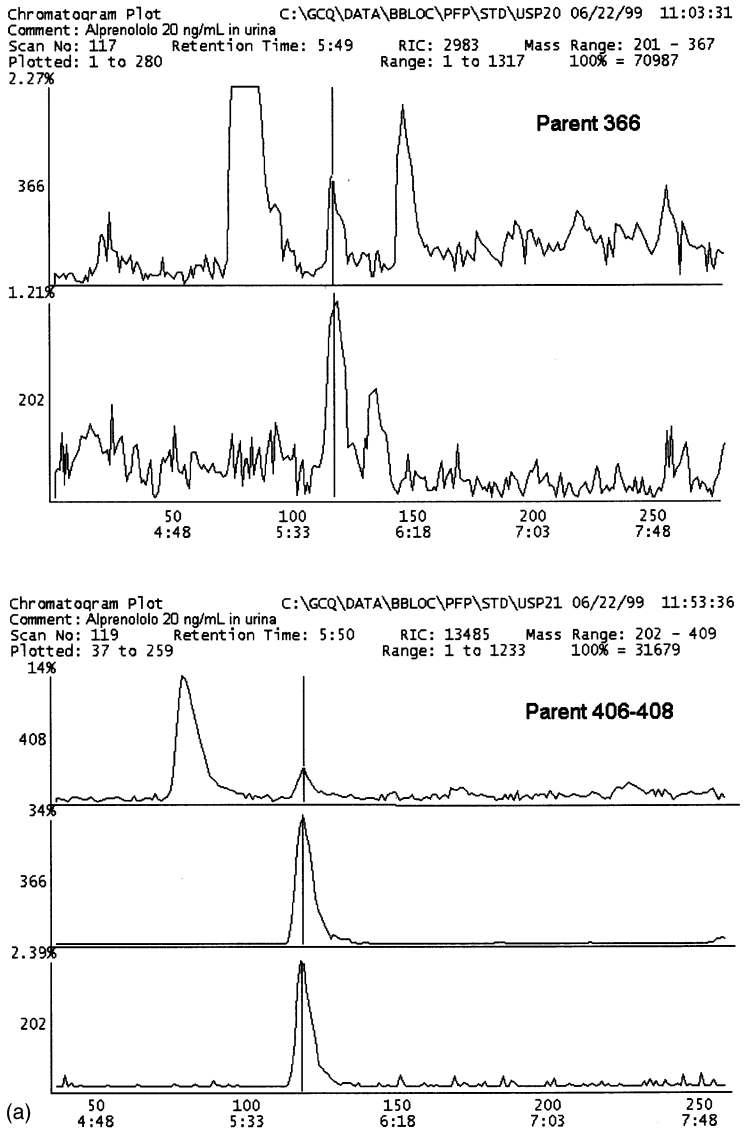

Fig. 5. Comparison between the GC-MS-MS-EI technique (a) (MS method # 1: above; MS method # 2: below) and the reference GC-MS-NCI technique (b). Data refer to the analysis of aprenolol 20 ng/ml in urine. Sample pretreatment and derivatization is the same in both cases.

cluding ultra short acting BB [19]) and to perform the subsequent confirmation analysis by the traditional GC-MS-NCI-SIM technique.

Current in progress are additional experiments carried out in order to:

1. Assess the detection limits for all BB included in the IOC list;
2. Compare the response of PFP- with other, different derivatives;
3. Extend the proposed approach to the analysis of other BB and of the corresponding urinary metabolites by specifically designed excretion studies.

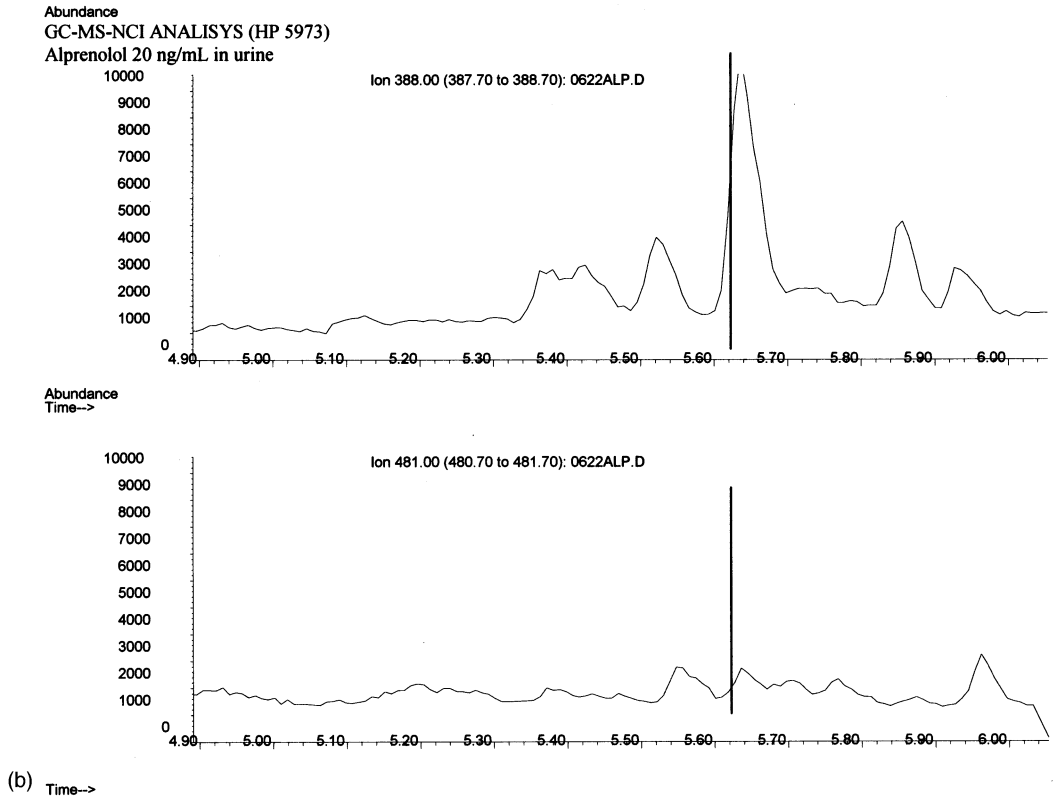

Fig. 5. (*Continued*)

The effectiveness of the method is under constant evaluation being applied, in comparison with the reference GC-MS-NCI technique, for the routine analysis of athletes urine carried out in the antidoping laboratory of Rome.

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