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SHORT COMMUNICATION

WILEY

Hair analysis for beta-blockers and calcium-channel blockers by using liquid chromatography-tandem mass spectrometry as a tool for monitoring adherence to antihypertensive therapy

Francesco Taus[1] | Rossella Gottardo[1] | Marco Ballotari[1] | Chiara Utzeri[1] | Franco Tagliaro[1,2]

[1]Unit of Forensic Medicine, Department of Diagnostics and Public Health, University of Verona, Verona, Italy

[2]World-Class Research Center "Digital biodesign and personalized healthcare", Sechenov First Moscow State Medical University, Moscow, Russia

Correspondence
Rossella Gottardo, Department of Diagnostics and Public Health, Unit of Forensic Medicine, University of Verona, Piazzale L.A. Scuro, 10-37134 Verona, Italy.
Email: rossella.gottardo@univr.it

Abstract

Adherence to therapy is the key to a successful therapeutic intervention, especially in cardiovascular diseases in which a lack of adherence may have serious consequences in terms morbidity and/or mortality. In this context, hair analysis can be an excellent tool to monitor adherence to therapy. Indeed, drugs present in blood are incorporated into the hair matrix, where drugs and metabolites can stay unaltered for a long time protected from metabolism and degradation. In the present study, a simple, specific, and sensitive ultra-high performance liquid-chromatography–tandem mass spectrometry (UHPLC-MS/MS) method set up to determine in human hair seven beta-blockers (viz., metoprolol, sotalol, labetalol, atenolol, nebivolol, bisoprolol, and nadolol) and two calcium-channel blockers (lercanidipine and amlodipine), which are widely prescribed to treat medium-to-severe hypertensive conditions. The optimized method was successfully validated in terms of accuracy, repeatability, reproducibility, matrix effect and extraction recovery. Moreover, the applicability of the method was evaluated by analyzing 34 real samples of hair obtained from patients under long-term therapy with calcium channel blockers and beta-blockers.

KEYWORDS
adherence to therapy, beta-blockers, calcium channel-blockers, hair analysis, mass spectrometry

1 | INTRODUCTION

The problem of scarce adherence to long-term medical treatments has been debated for years, as it results from the inspired document of the World Health Organization (WHO) "Adherence to long-term therapies. Evidence for action."[1]

In this document, WHO points out that adherence to long-term therapy in developed countries averages 50%, rating even lower in developing countries. As consequences of scarce adherence, poor health outcomes and increased healthcare costs are observed.

Thus, increasing the effectiveness of adherence interventions can have a much greater impact on population health than any improvement in specific medical treatments.

Unfortunately, to date, the assessment of adherence to therapy may rely on tools of poor effectiveness, which include interviews/questionnaires, counting doses taken by the patient, and measuring a

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marker added to drugs.[2] Even the analysis of the active ingredients/metabolites in biological fluids may provide data of scarce usefulness, because of their dependence on pharmacokinetics and metabolism.[3,4]

The use of hair analysis monitoring drug abuse histories dates back to the 1980s, while monitoring the intake of medicinal drugs by using hair analysis represents a relatively new approach, with limited literature data and substantially no evidence from controlled studies.

On these grounds, the hypothesis of the present projects is using hair as an alternative biological matrix to monitor adherence to long-term pharmacological treatments. Hair, as other in keratinized tissues, based on a sound experience in forensic toxicology, has proved to be suitable for retrospective analytical investigations.[5] The embedding of xenobiotics at the root level, the known and relatively constant speed of hair growth and their protection from degradation and metabolism by the hair matrix are the basic evidence that support the suitability of hair analysis for studying retrospectively the intake of xenobiotics.

Strangely enough, hair analysis in the field of forensic toxicology is extensively used to monitor drug abuse and in the investigation of drug-facilitated crimes, whereas it has only occasionally been proposed to monitor adherence to therapy[6–8] of isoniazid,[9] antiretrovirals,[10] drugs for the treatment of headache,[11] and inhaler drug therapy.[12]

To date, to the best of our knowledge, only two papers reported the use of hair analysis for the determination of cardiovascular drugs (viz., beta-blockers and hydrochlorothiazide), but in a context of doping control.[13,14]

On these bases, the present work aimed to verify the possibility to used liquid chromatography hyphenated with mass spectrometry to quantitatively determine antihypertensive drugs (particularly beta-blockers and calcium channel blockers) in the hair of subjects undergoing chronic treatments, under a rigorous validation protocol.

The study includes seven beta-blockers (viz., atenolol, bisoprolol, labetalol, metoprolol, sotalol, nebivolol, and nadolol) and two calcium channel blockers (amlodipine and lercanidipine). To this aim, an original method based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been developed and validated. The practical applicability of the method was also verified by analyzing 34 authentic hair samples collected from subjects under chronic therapy with those cardiovascular drugs.

2 | MATERIAL AND METHODS

2.1 | Chemicals and reagents

Formic acid for LC-MS was obtained from Merck KGaA (Darmstadt, Germany). Methanol, acetonitrile, dichloromethane, isopropanol, and hydrochloric acid were purchased from VWR Chemicals (Fontenay-Sous-Bois, France). Disodium hydrogen orthophosphate and ammonia solution 30% were provided by Carlo Erba Reagenti (Milan, Italy).

Alprenolol, atenolol, metoprolol, and nifedipine were purchased by Merck KGaA (Darmstadt, Germany). Sotalol and nebivolol was provided by ABC Farmaceutici (Ivrea, Italy). Bisoprolol and lercanidipine were purchased by Sandoz (Origgio, Italy). Nadolol and amlodipine were obtained from Pfizer (Freiburg, Germany).

Stock solutions of the standards were dissolved in methanol at concentrations of 1 mg/ml and kept frozen in darkness at −20°C. Because of the known photosensitivity of calcium channel blockers, special care to minimize light exposure was adopted for these compounds.[15] Appropriate amounts of the stock solutions were added to blank hair to obtain hair samples with known concentrations of the drugs of interest.

HyperSep Verify-CX extraction cartridges (130 mg, 6 ml) were purchased from Thermo Fisher (Waltham, Massachusetts, USA).

Ultrapure water was produced with a model PureLab Chorus 1 Complete water purification system (Elga Veolia Lane End, High Wycombe, UK).

2.2 | Hair specimens

Blank hair for preparation of quality controls (QC) and calibration standards were obtained from well-known subjects abstinent from any drugs for the previous 6 months. Hair samples from patients ($n = 34$) were collected from subjects undergoing long-term therapy with beta-blockers and calcium channel blockers at different dosages for the treatment of hypertension.

Hair collection was performed by cutting with scissors at the scalp level posterior vertex hairs with an average length of 4–5 cm. Assuming a hair growth rate of 1 cm/month, this procedure allowed us to monitor the drug intake over a period of 4–5 months before sample collection.

2.3 | Sample pre-treatment

Hair samples (25 mg) were weighed and washed twice with dichloromethane to remove any potential contaminants present on the hair surface. Hair blanks were spiked with a test mixture of atenolol, bisoprolol, labetalol, metoprolol, sotalol, nebivolol, nadolol, amlodipine, nifedipine, and lercanidipine, at suitable concentrations to mimic drug concentrations found in patients (0.5–10 ng/mg for beta-blockers and 2–40 ng/mg for calcium channel blockers).[13] Alprenolol (the used internal standard, IS) was added to all samples at a concentration corresponding to 4 ng/mg of drug in hair.

Subsequently, the hair was cut into small fragments and incubated overnight in 1 ml of 0.1-M HCl at 45°C. Finally, the incubation mixture was neutralized with equimolar amounts of NaOH and extracted by using a HyperSep Verify-CX cartridge.

The SPE cartridges were initially activated by using 2-ml methanol and then rinsed with 2 ml of 0.1-M phosphate buffer solution at pH 6 before loading the sample. The columns were sequentially washed with 2-ml deionized water, 3-ml HCl 0.1 M, and 4-ml methanol. The analytes were then eluted with 2 ml of dichloromethane/isopropanol

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(80:20, v/v) containing 2% ammonia solution. The eluate was then dried under a nitrogen stream and reconstituted in 200 µl of mobile phase. Finally, 5 µl of the solution were injected in the LC-MS/MS system.

2.4 | Instrument conditions

LC-ESI-QqQ MS analyses were performed on a model 1290 Infinity HPLC–6460 MS (Agilent, Milford, MA, USA) fitted with a Jetstream

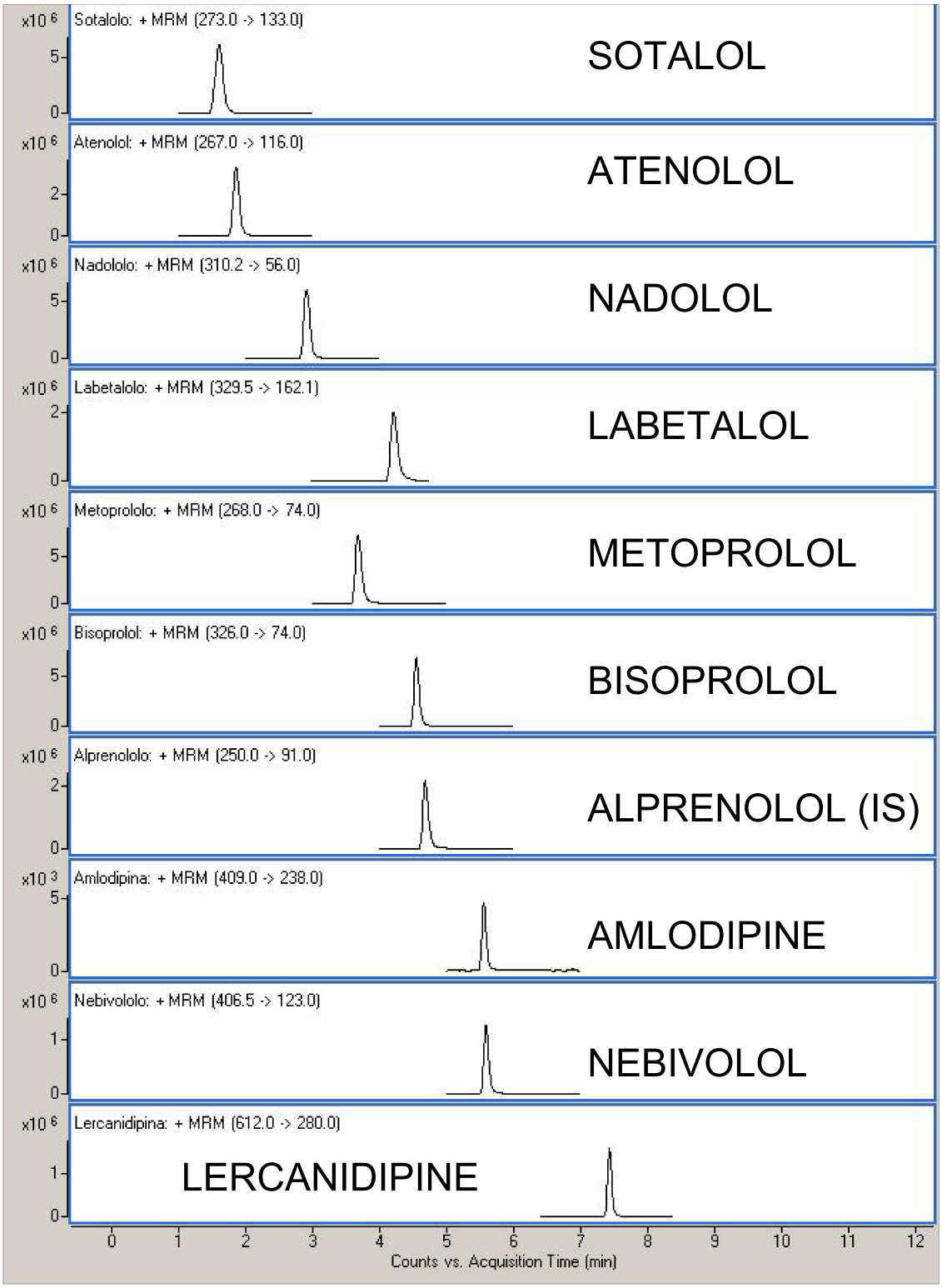

FIGURE 1 Extract ion chromatograms of a mixture of the studied analytes each at the individual concentration of 10 ng/mg (IS: alprenolol) [Colour figure can be viewed at wileyonlinelibrary.com]

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electrospray ionization source in positive acquisition mode. Separations were achieved by using model Eclipse Plus C18 (1.8 μm; 2,1 × 100 mm) column (Agilent) at 60°C. The injection volume was 5 μl. Mobile phase A consisted of 5-mM aqueous ammonium formate and 0.01% formic acid in water and acetonitrile, whereas mobile phase B was composed of methanol/acetonitrile (90/10, v/v) containing 0.01% formic acid.

The separation was carried out in gradient mode at a flow rate of 0.5 ml/min, with the following elution program: 0–1 min 5% B, 1–8.5 min linear gradient from 5 to 95% B; held for 2 min at 95% B, before re-equilibration at 5% B.

The mass spectrometer was operated with a drying gas flow of 8 L/min, nebulizer pressure of 30 psi, drying gas temperature of 320°C, capillary voltage of 3,750 V.

The sheath gas heater and sheath gas flow were 355°C and 12 L/min, respectively. The optimal MRM transitions, fragmentor, and collision energies for the different compounds were determined by direct infusion of pure standards into the ion source of a standard solution of each compound at a concentration of 1 μg/ml in 50/50 v/v mobile phase A and B. Specific multiple reaction monitoring (MRM) transitions were chosen for each compound. Furthermore, to improve the selectivity and sensitivity of the method, collision energy and fragmentor voltage were optimized.

The precursor ions and the ionic transitions of each analyte were assessed by direct infusion precursor ions and the optimized parameters are summarized in Table S1.

A typical chromatogram obtained by injecting a standard mixture of the tested compounds, each at a concentration of 10 ng/mg is depicted in Figure 1.

Data acquisition and data handling were carried out by using the software Mass Hunter Data Analysis (Agilent).

2.5 | Validation procedure

The method was evaluated in terms of linearity, sensitivity, selectivity, accuracy, precision, matrix effect, and extraction recovery according to the international guidelines.[16]

The linearity of response was assessed for each compound for the concentration range of 0.5–10.0 ng/mg for beta-blockers and 2.0–40.0 ng/mg for calcium-channel blockers. The LOD's were calculated as the concentrations determined with S/N = 3, whereas LLOQ corresponded to the concentrations at which inaccuracy and imprecision of determination was less than 20%.

Selectivity was assessed by testing for possible interfering compounds in 20 different hair samples collected from healthy volunteers who declared not to have taken any drug in the previous 6 months.

Intraday and day-to-day precision, expressed as relative standard deviation (% RSD), were calculated at the QC levels, as average of $n = 6$ repeated injections. Analytical accuracy, expressed as the deviation from the nominal value, was calculated at the same QC levels used for precision assessment. For this purpose, three QC levels (low, medium, high) were prepared by spiking blank hair with standard solution to mimic drug concentrations in hair of 2.10 and 40 ng/mg for calcium channel blockers and 0.5, 2 and 10 ng/mg for beta-blockers. The injection of the QC levels was repeated six times in three different days.

Matrix effects and extraction recovery were evaluated according to the procedure proposed by Matuszwski et al.[17] Briefly, matrix effects were assessed by comparing the detector response to an analyte in pure solution with that resulting from the analyte added to an extract of a blank biological sample that has undergone the standard sample preparation process. In the present work, the peaks of pure standard injected in pure solvent (A) were compared with those resulting from the injection of in a blank blood extract added with the same analytes to achieve the same analyte concentrations (B). The ratio B/Ax100 was defined as the absolute matrix effect (ME %). Signal enhancement is reported if the ME% is >100 and signal suppression if the value is <100.

Extraction recovery (RE) was instead calculated by comparing the mean peak areas of the analytes from the injection of extracted samples (spiked before extraction) with that of samples spiked after extraction. The matrix effect and extraction recovery evaluation were performed in triplicate for each QC tested concentration.

3 | RESULTS AND DISCUSSION

3.1 | Method validation

In the present study, linearity was evaluated in the range between 0.2 and 10.0 ng/mg for beta-blockers and 2.0 and 40.0 ng/mg for calcium-channel inhibitors. As shown in Table S2, linearity was satisfactory for all the tested compounds, being the regression coefficients R always higher than ≥0.98.

LODs and LLOQs were 0.2 and 0.5 ng/mg for beta-blockers and 1.0 and 2.0 ng/mg for calcium-channel blockers, respectively.

No possibly interfering peaks with the compounds of interest were observed in the investigation of method selectivity.

The intraday and interday precision of the new analytical method was assessed by serial injections of samples containing all the studied analytes on the same day and on three different nonconsecutive days. For each sample, six repetitions were carried out. Precision was studied at three different concentrations (1, 5, and 10 ng/mg for beta-blockers; 5, 20, and 40 ng/mg for calcium channel-blockers).

The accuracy of the method was evaluated in terms of bias, which was measured as a percentage deviation from the nominal concentration levels. In the present experiments, bias was always within ±15% for all concentrations tested.

Bias and precision validation data are summarized in Table S3.

In this study, the matrix effect was within acceptable values (±25%) for all analytes. The extraction recovery was always higher than 85%.

3.2 | Application to real cases

The applicability of the method to the study of patients undergoing long-term therapy with the mentioned medicines was verified by

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analyzing $n = 34$ hair samples collected from 25 patients. Examples of the chromatograms from these authentic hair samples are reported in Figure 2. Out of the 34 samples, in two cases, the detected compound differs from the declared beta-blocker while three samples were negative for the expected drug.

In three cases, the expected drugs (atenolol or amlodipine) were present only in traces (in concentration between the LOD and the LOQ).

In general, the drug concentrations found are shown in Table S4. For example, atenolol ranged from 0.62 to 3.99 ng/mg (median 1.6), amlodipine from 2 to 35.4 ng/mg (median 15.06), and bisoprolol from 1.30 to 26.7 ng/mg (median 3.3).

In the present study, a great variability of the drug concentration was found. As already known, drug incorporation into hair can be affected by many factors, including external contamination, hair color or melanin content,[18] lipophilicity of the drug, and cosmetic treatment of hair.[19,20]

Among all, hair pigmentation is a critical factor in the interpretation of drug concentration in hair, because it can influence the incorporation of drug into the matrix. Particularly for basic drugs, darkly pigmented hair can bind higher amounts of drugs than lightly pigmented hair, because of the higher melanin content.

In the present study, the hair samples tested were either brown/dark colored or grizzle. Only two hair samples were dyed. As expected, amlodipine concentrations seem to be lower in grizzle hair rather than in brown hair, likely due to the basic nature of this compound.

In the case of atenolol, the lowest concentrations were found in the two subjects with dyed hair. Unfortunately, due to the limited studied samples, it was not possible to establish whether hair color or cosmetic treatments can affect and to what extent the incorporation of the studied compounds in hair.

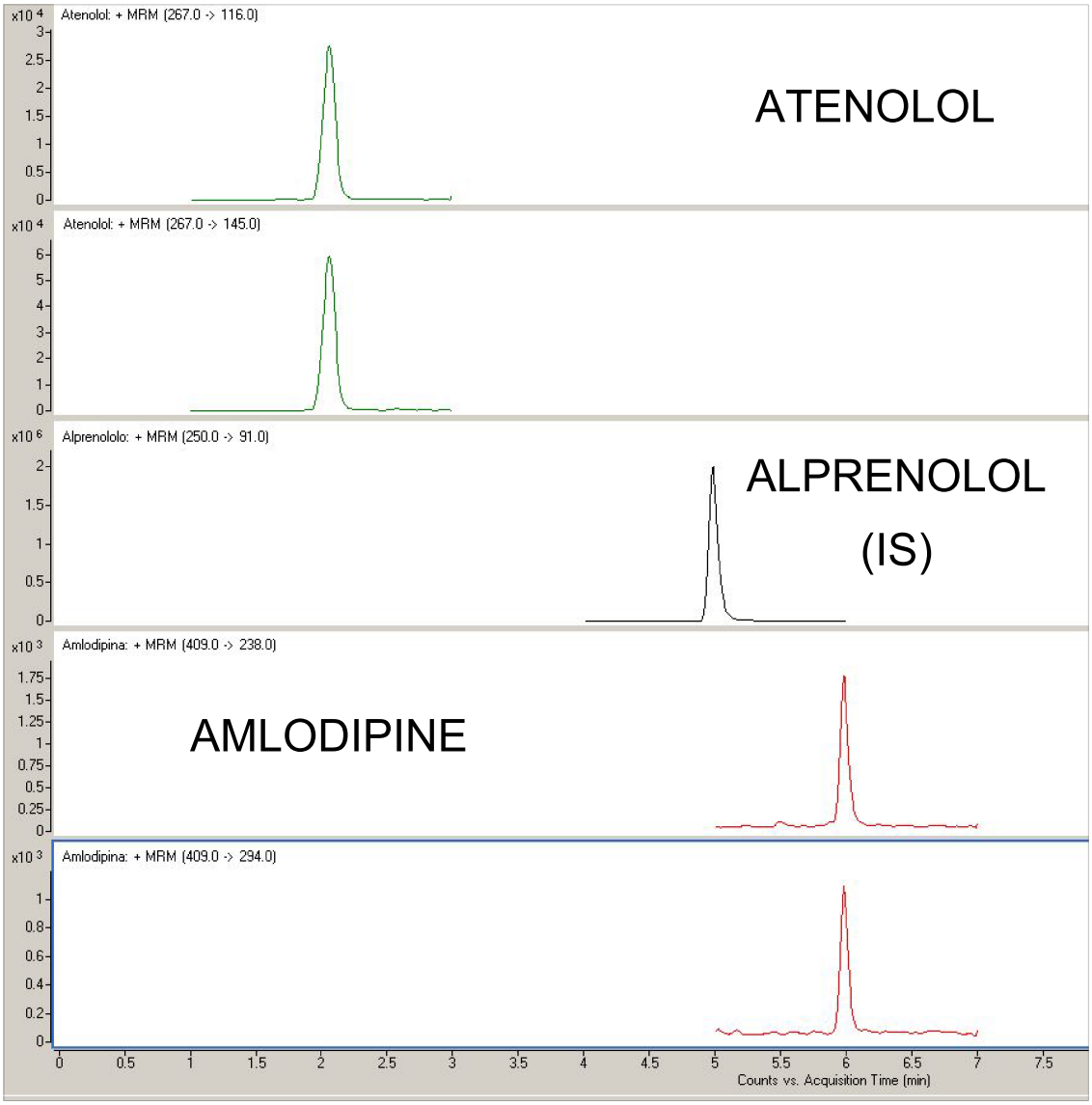

FIGURE 2 Extract ion chromatograms of an authentic hair sample containing atenolol and amlodipine at the concentration of 0.62 and 13.9 ng/mg, respectively (IS: alprenolol) [Colour figure can be viewed at wileyonlinelibrary.com]

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4 | CONCLUSIONS

The method here described, aimed at the quantitative determination of seven beta-blockers and two calcium-channel blockers in human hair, has been fully validated according to international guidelines, showing good sensitivity and selectivity with negligible matrix effects and very good linearity, accuracy, precision, and extraction recovery. Also, its preliminary application to the analysis of 34 authentic hair samples collected from randomly selected patients under treatment with the most common of these medicines was successful, because only two of them resulted "negative" for the expected drug, which can tentatively be ascribed to poor compliance to therapy.

To the best of our knowledge, this is the first study reporting the concentrations of beta-blockers and calcium channel antagonists in the hair of patients in the frame of monitoring adherence to therapy.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTION

Francesco Taus: data acquisition, original draft preparation; Marco Ballotari: Investigation; Chiara Utzeri: data acquisition; Franco Tagliaro: strategic design, writing-review & editing, fund acquisition, Rossella Gottardo: conceptualization, methodology, original draft preparation, final editing.

DATA AVAILABILITY STATEMENT

Data available on request from the authors

ORCID

Francesco Taus https://orcid.org/0000-0003-4728-6686
Rossella Gottardo https://orcid.org/0000-0001-5997-1723
Franco Tagliaro https://orcid.org/0000-0002-2187-9128

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SUPPORTING INFORMATION

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