**Detection of the synthetic peptide ipamorelin in dried blood spots by means of UHPLC-HRMS**

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**Abstract**

Growth hormone-releasing peptides (GHRPs) are synthetic peptides that stimulate the release of growth hormones from the hypothalamus and pituitary gland. Among these, ipamorelin is a highly selective, potent and effective pentapeptide belonging to the class of GH secretagogues (GHS). Because of clinical monitoring and the potential abuse by athletes in amateur and elite sports, detection methods able to identify GHRPs in biological samples are increasingly needed. As an alternative to conventional procedures, testing of Dried Blood Spots (DBS) is gaining increasing attention, since its low volume sampling is easy and scarcely invasive. In this study, a UHPLC-HRMS method was developed and validated for the screening of ipamorelin in DBS. Ipamorelin was extracted from DBS by liquid extraction and ultrasonic treatment, then detected by the acquisition of high-resolution fragmentation spectra. The method proved satisfactory in terms of sensitivity, specificity, linearity, recovery, precision and ion suppression/enhancement effects. The mass error of the precursor and fragment ions was lower than 5 ppm. Since ipamorelin was successfully detected in DBS with a detection limit of 2.5 ng/mL, the combination of DBS and HRMS proved to be a suitable and effective method to detect small peptides in blood at low levels. This approach looks promising for targeted and untargeted analysis in clinical monitoring and sport drug testing, especially when unknown synthetic peptides represent the target analytes.

1. **Introduction**

Growth hormone-releasing peptides (GHRPs) are synthetic peptides that stimulate the release of the growth hormone (GH) from the hypothalamus and pituitary gland and modulate food intake, sleep, and cardiac tone [1]. As a consequence, these peptides have received considerable attention in sports drug testing and are included in the World Anti-Doping Agency (WADA) Prohibited List [2]. Among GHRPs, ipamorelin is a potent and selective pentapeptide that belongs to the class of growth hormone secretagogues (GHS). Ipamorelin displays high growth hormone potency and efficacy both *in vitro* and *in vivo*. Unlike the others GHRPs, ipamorelin produces an increase of the GH plasma level without increasing the adrenocorticotropin (ACTH) and cortisol levels [3]. For clinical purposes, ipamorelin was administered to increase gastrointestinal mobility in ileum diseases [4]. In a second study, ipamorelin was tested as an alternative to the direct administration of GH in the treatment of children with growth delay [5]. Although ipamorelin and other GHRPs have not received approval for clinical use, several peptides (including ipamorelin) and analogues were identified in black market products [6–9], suggesting their potential misuse for doping purposes.

In sport drug testing, several methods for the detection of GHRP in urine and blood or plasma have been developed [10]. These methods commonly use solid phase extraction (SPE) based on cationic weak exchange stationary phase or protein precipitation, sometimes followed by immunoaffinity purification, followed by liquid chromatography separation coupled to tandem mass spectrometry and/or high resolution mass spectrometry detection [11–20].

Currently, sampling of blood specimens in sports drug testing is considered invasive and require the presence of trained personnel for venous blood sampling. Furthermore, samples shipment and storage at controlled temperature, limited stability and timely analysis, all represent essential prerogatives. Compliance with these requirements implies considerable cost per sample, especially when a limited number of specimens has to be collected. The use of alternative matrices and procedures, including the collection of dried blood spots (DBS), could overcome these limitations, increasing the number of tested blood samples whiledecreasing the cost of sampling.

DBS is a micro-sampling technique involving the deposit of a minimal volume of capillary blood on a cellulose adsorbent card that can be easily stored in a fresh and dark place for a long time to preserve the analyte stability. The speed and ease of sampling, its low invasiveness and intrusiveness, and the low transportation and storage costs, are among the advantages suggesting the DBS application in several investigation fields, such as new-borns screening [21], therapeutic drug monitoring (TDM) [22,23], clinical chemistry [24], toxicology [25–27], and toxicokinetic [28,29]. In sport drug testing, DBS was taken into consideration as an alternative matrix only recently, and its increasing potential to support result management processes in routine testing has been discussed in the latest literature [10,30]. Some of the existing doping control assays on DBS aim at low molecular mass analytes [31,32] and a limited number of peptide drugs. Recently, Lange et al. developed a LC-HRMS method for the quantitative detection of 46 low molecular mass (<2 kDa) peptide/non-peptide drugs and drug candidates, after a fully automated DBS sample preparation [33]. LC-MS/MS methods for the detection of paginesatide and Synachten were developed by Moller et al. [34] and Tretzel et al. [35], respectively. A recent screening method for insulin and its analogues was developed by Thomas et al., [36] while a previous study demonstrated the detection of IGF-1 in DBS by LC-MS [37]. Recently, Lange and co-workers determined the TGF-β signaling inhibitors sotatercept and luspatercept and the ActRIIA/B-antibody bimagrumab in DBS by LC-HRMS [38].

In the present study, a comprehensive workflow for the specific and sensitive detection of ipamorelin in DBS is presented. Our aim was to develop a simple, suitable and effective procedure based on fast sample extraction followed by a UHPLC-HRMS detection, in order to detect small peptides in blood at low levels.

1. **Materials and methods**
   1. **Chemicals and reagents**

Acetonitrile, citric acid, ipamorelin acetate (98%), MRFA (tetrapeptide Met-Arg-Phe-Ala) and DBS Whatman FTA DMPK-C papers were purchased from Merck Italia (Milan, Italy). Formic acid was supplied by Fisher Scientific Italia (Rodano, Milan, Italy). Ultrapure water for UHPLC use was obtained from a Milli-Q® equipment (Millipore, Bedford, MA, USA). Negative blood was obtained from 4 volunteer subjects. Two stock standard solutions of ipamorelin and MRFA were prepared in ultrapure water at the concentration of 1 mg/mL and stored at -20°C until use.

* 1. **Analytical protocol**

Prior to their use, DBS cards were conditioned using 10 µL of 0.5 M citric acid and then dried at room temperature for 1 hour [36]. 40 µL of human whole blood were spotted onto the DBS card and dried at room temperature for 1 hour. Each spot was spiked with 8 µL of a 1 ng/mL solution of internal standard (MRFA) and dried at room temperature for 30 minutes. The circular spots were carefully cut and transferred into a 1.5 mL Eppendorf tube. 200 µL of a 50:50 water: acetonitrile solution with 10% formic acid was added to each tube and centrifuged at 13,500 rpm for 5 minutes. Each tube was then shaken at room temperature for 30 minutes prior of being centrifuged again at 13,500 rpm for 10 minutes. 5 µL of the supernatant was injected into the UHPLC system. UHPLC separation was performed on a Phenomenex C18 column (100 × 2.1 mm, 1.7 µm) held at 40ºC on the SCIEX Exion LC™ AC System (Sciex, Darmstadt, Germany). Mobile phases were 5mM formic acid in water (Phase A), and acetonitrile with 5mM formic acid (Phase B). The LC flow rate was 0.5 mL/min and the total run time was 7 min. MS and MS/MS data were collected for each sample using QTOF MS/MS acquisition on the SCIEX X500R QTOF system operating in the positive ESI mode. The first phase of data acquisition was a TOF-MS scan (230-800 Da), with accumulation time set at 0.25 s, collision energy (CE) of 10 V, temperature of the source set at 300 °C and spray voltage (SV) set at 2200 V. The declustering potential (DP) was 50 V. The second step of data acquisition was a TOF-MS/MS experiment which monitored all the fragments in a mass range 50 to 720 Da, with an accumulation time of 0.3 s. The CE was 35 V and the DP used was 25 V. The mass spectrometer acquired full scan and MS/MS data at a resolving power of 35,000 FWHM. Calibration was carried out according to the manufacturer’s specification ensuring a mass error below 5 ppm. Data were acquired using SCIEX OS Software 1.5. Each extracted sample was injected in triplicate over the course of three consecutive days.

* 1. **Method Validation**

The validation process was conducted with an optimized procedure, requiring the preparation of three replicates of the calibration curves for the targeted compounds in three consecutive days for a total of nine calibration curves [39,40]. The main validation parameters were determined from these data, including linearity range and calibration model, selectivity, specificity, limit of detection (LOD), limit of quantification (LOQ), intra and inter-assay precision and accuracy. The linearity was evaluated within the concentration range of 5–100 ng/mL with calibration points at 5, 10, 25, 50, and 100 ng/mL. The best calibration model was evaluated using the RStudio routine developed by Desharnais et al. [41,42], which included the study of homoscedasticity (from which the introduction of appropriate calibration weights was deduced) and of the order of the calibration model (linear or quadratic). The LOD and LOQ were estimated by means of the Hubaux-Vos’ algorithm [43] applied in the linear dynamic range and corrected for the heteroscedasticity weights [39,40].

To determine selectivity and specificity, the signal-to-noise ratio (S/N > 3) was measured on the selected ion chromatograms at the expected retention times for all the analytes of interest. The presence of interfering peaks around the retention time of the analytes was examined. Intra- and inter-day precision and accuracy were evaluated using two dedicated back-calculation approaches, as described elsewhere [39,40]. Optimal percent coefficient of variation (CV%) and percent bias were expected to lie within ±15%, while results within ±25% were considered satisfactory. Retention time repeatability was verified at all concentration levels. Carry-over effect was evaluated by injecting one blank extracts after the highest point of each calibration curve: if S/N ratio was lower than 3 in each ion chromatogram the carry-over effect was considered negligible.

The matrix effect was estimated at high, low, and intermediate concentration levels by comparing the experimental results obtained from spiked whole blood and neat solutions, both spiked after the extraction step. The matrix effect for each target analyte was expressed as the percentage ratio between the two measured concentrations. The extraction recovery was determined by comparing the experimental results from whole blood samples spiked respectively before and after the extraction step and was expressed as the percentage ratio between the two quantified concentrations.

1. **Results and discussion**
   1. **UHPLC-HRMS identification**

The positive electrospray ionization of ipamorelin results in the predominant doubly protonated ion at m/z 356.7001. Accordingly, all MS experiments were optimized on the precursor ion at 356.7 m/z, corresponding to the [M+2H]2+ ion. Following the collision induced dissociation (CID) experiments of the precursor ion, the fragment ions derived from the y-series at m/z 146.1292 (y1), 490. 2820 (y3), 627.3396 (y4) and the b-series at 223.1191 (b2), 420.2041 (b3) and 567.2720 were observed. The chemical structure of ipamorelin, the schematic peptide fragmentation and the list of the observed ion fragments are reported in Figure 1. The product ion mass spectrum of ipamorelin, together with the proposed fragmentation pattern, are available as supplementary material (Figures S1 and S2) .The specificity of the method was demonstrated by the absence of interfering signals in the extracted ion chromatogram of ipamorelin at the expected retention time. The choice of the experimental parameters fulfilled the recommended chromatographic and mass-spectrometric identification criteria for target analytes in biological fluids [44]. The identity of ipamorelin was verified by three diagnostic fragment ions extracted from the MS/MS spectrum observed at 129.1022, 223.1191, and 420.2041 Da and assigned to the y1-NH3, b2 and b3 peptide fragments of ipamorelin, respectively, in accordance with a previous study [16]. Mass error of all characteristic fragment ions was lower than 5 ppm. Figure 2 shows the extracted ion chromatogram (XIC) of dried blood spots sample fortified with 5 ng/mL of ipamorelin together with the TOF-MS spectrum reporting the ipamorelin precursor ion [M+2H]2+ at m/z 356.7 and the corresponding MS/MS product ion spectrum. Table 1 reports the experimental mass errors for ipamorelin precursor ion [M+2H]2+ (theoretical value 356.7001 Da) and fragment ions (theoretical values 129.1022, 223.1190, and 420.2030 Da) obtained from the experiments performed on dried blood spots sample fortified with 5, 25, and 100 ng/mL. These data, collected during three consecutive days, showed a high level of reproducibility across the three concentrations. In particular, the exact masses of the three peptide fragments of ipamorelin observed in the MS/MS spectra were consistent over the course of three days. Therefore, the analytical workflow produced reproducible and accurate results for each injection of every batch, demonstrating the robustness and stability of the method.

* 1. **Validation of the analytical method**

The results of the validation experiments for DBS samples fortified with ipamorelin at three concentrations (5, 25 and 100 ng/mL) are reported in Table 2. From the F-Test integrated in the RStudio routine, the calibration model resulted heteroscedastic (p-value = 1.1 × 10−11) and linear (p-value = 5.1 × 10−1). The LOD, calculated using the Hubaux-Vos algorithm, was equal to 2.5 ng/mL and the LOQ was equal to 5.0 ng/mL, corresponding to the lowest calibration level of the analytical method. These results show that the presented workflow allows adequate sensitivity for the detection of ipamorelin extracted from DBS. In particular, the low ng/mL detection range seems appropriate for routine applications. In clinical monitoring, the observed blood levels of ipamorelin 10 hours after the infusion of 14.04, 42.12, 84.27 and 140.45 nmol/kg doses (around 10, 30, 60 and 100 μg/kg) was found in the range between 4 and 100 nmol/L (around 3 and 70 ng/mL) [45]. In antidoping controls, the 2 ng/mL level represents the minimum requirement limit for ipamorelin in urine [46], but at the moment specific recommendation for DBS (and blood) have not been set. Inter-day and intra-day precision (expressed as percent variation coefficient, CV%) and accuracy (expressed as bias%) were found below 25% and 20%, respectively, for the calibrators at 5, 25 and 100 ng/mL concentration. The only exception was the intra-day precision at 5 ng/mL, which exceeded (32.7%) the predetermined limit of 25%. In general, the data collected during three repetitions in three consecutive days and across three concentrations level showed satisfactory reproducibility, again proving the robustness of the overall workflow. The extraction recovery tested at 5, 25 and 100 ng/mL concentration was found in the 39-49% interval, as expected for a DBS test, while the experiments aimed to evaluate matrix effects revealed the occurrence of ion enhancement at the lowest concentration (+48%), while this effect appeared considerably reduced at higher calibration levels (+11%). Potential application to sport doping testing will require further studies to address to the stability in different conditions of time and temperature, in order to simulate i) the cards transfer from the sampling site (e.g. the sport event location) to the testing laboratory, ii) the cards short-term storage before the analysis, and iii) the cards long-term storage before the counter analysis, in case the individual (i.e. the athlete) tested positive requires to challenge the positive result.

1. **Conclusions**

A novel and comprehensive workflow for the detection of ipamorelin from DBS was successfully developed using an UHPLC-QTOF-HRMS approach. The combination of a rapid sample extraction procedure and a highly selective MS/MS acquisition method enabled sensitive detection of ipamorelin spiked at low ng/mL concentration in DBS. The use of a HRMS instrument allowed the univocal identification of the analyte of interest and provided at the same time the chance of conducting a reliable retrospective analysis. In this context, ipamorelin could represent a model compound of the classes of GHRPs present in the WADA Prohibited List, from which the present procedure can be adapted to include a larger panel of analytes. The interest in the use of DBS in routine doping control is steadily increasing, as shown by recent researches funded by WADA on this topic [47]. The sample collection is modestly invasive and cost-effective assuring to DBS testing a promising future in sports drug checking as a method complementary to urine analysis. This is likely to allow a significant increase of in/out-of competition testing within the existing monitoring programs. Unlike blood or urine samples, the DBS cards do not require to be maintained at controlled (cold) temperatures, offering the benefit of doable sampling in remote world areas. Finally, the possibility of expanding the panel of target analytes, together with the feasible automation of sample treatment processes, will allow in the future to increase the number of tests on a larger number of molecules in a short time, drastically reducing the costs of analysis.

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**Table 1**: Experimental masses and mass errors of precursor and fragment ions of ipamorelin observed after TOF-MS and TOF-MS/MS experiments on DBS spiked with blood at three different concentrations of analyte (5, 25 and 100 ng/mL).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Concentration**  **(ng/mL)** | **Observed precursor ion (m/z) a** | **Mass error (ppm)** | **Observed fragments (m/z) b** | **Mass error (ppm)** |
| 5 | 356.7002 | 0.3 | 129.1016  223.1189  420.2038 | -4.6  -0.9  1.9 |
| 25 | 356.7003 | 0.6 | 129.1024  223.1194  420.2031 | 1.5  1.3  0.2 |
| 100 | 356.6998 | -0.8 | 129.1021  223.1195  420.2042 | -0.8  1.8  2.9 |
| a theoretical precursor ion: 356.7001 m/z  b theoretical fragment ions: 129.1022 m/z, 223.1191 m/z, 420.2030 m/z | | | | |

Table 2: Summary of validation data: calibration model, precision, accuracy, recovery and matrix effect.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Ipamorelin** | | | | | | | | | | | |
| **Calibration model** | |  | **Concentration**  **(ng/mL)** | **Precision** | |  | **Accuracy** | |  | **Recovery (%)** | **Matrix effect (%)** |
| **Equation** | **R2** |  | **Intra-day (bias%)** | **Inter-day (bias%)** |  | **Intra-day (CV%)** | **Inter-day (CV%)** |  |
| 14.13x + 0.10 | 0.99499 |  | 5 | 32.7 | 18.5 |  | 15.2 | 7.2 |  | 46 | +48 |
|  | 25 | 10.6 | 23.5 |  | -19.4 | -19.3 |  | 49 | +11 |
|  | 100 | 15.7 | 20.1 |  | -12.8 | 5.9 |  | 39 | +11 |

**Figure 1:** Chemical structure of ipamorelin, its schematic peptide fragmentation and the list of the observed ion fragments.

**Figure 2**: Extracted ion chromatogram of ipamorelin in DBS at 5 ng/mL and the corresponding high-resolution mass spectrum of the protonated molecule [M+2H]2+at m/z 356.7. The product ion mass spectrum of the precursor ion at m/z 356.7 is shown below.



