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Revealing of endogenous Marinobufagin by an ultra-specific and sensitive UHPLC-MS/MS assay in pregnant women

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ABSTRACT

Marinobufagenin (MBG) is a bufadienolide cardiac inotrope implicated in volume expansion-mediated hypertensive states including essential hypertension and preeclampsia (PE). Endogenous MBG is an inhibitor of the α1-isoform of Na^+,K^+-ATPase with vasoconstrictive and cardiotonic properties, causing hypertension and natriuresis.

Elevated endogenous MBG-like material levels have been described by immunoassays in salt-sensitive pregnant and preeclamptic rats as well as in preeclamptic human patients. The rise of endogenous MBG-like material appears prior the development of the main symptoms of PE, leading us to consider MBG as one of the potential biomarkers for PE.

The weak specificity and the high variability of the published immunoassays gives no certification about endogenous MBG existence. This led us to set-up a highly specific and sensitive analytical method to detect MBG in plasma at low levels relying on liquid chromatography combined to mass spectrometry (UHPLC-MS/MS) with recording of 7 highly specific MRM transitions for MBG. Pure MBG standard used in the method development was obtained by purification from the *Bufo marinus* toad venom. d_3–25-hydroxyvitamin D3 was used as internal standard. An increasing organic gradient with mobile phase A and B composed of 97:3 (v/v) H_2O: MeOH and 50:45:5 (v/v/v) MeOH:IPA:H_2O at pH 4.5 respectively was used on a Pursuit 3 PFP column (100 mm × 3 mm; 3 μm) to allow elution and separation of the plasmatic compounds. Chromatographic analyses of plasma samples were preceded by a precipitation of proteins pretreatment.

The developed UHPLC-MS/MS assay has been applied to early-pregnant women plasma samples allowing us to investigate MBG plasma levels. Thanks to the high specificity of the assay we were able to authenticate and certify the presence of endogenous MBG in early-pregnant women plasma with the use of the 7 selected specific mass transitions. These pioneering preliminary results are giving a promising perspective for early preeclampsia risk assessment in pregnant women.

1. Introduction

Marinobufagenin (MBG) is a cardiotonic steroid belonging to the family of bufadienolides (Fig. 1). These compounds consist of steroid frame with a 6-membered lactone ring in the C17 position. MBG is mainly known as one of the major components in the venom of the toad species *Bufo marinus* [1]. In amphibians, bufadienolide compounds are synthesized in response to environmental changes in salinity and hydric equilibrium. They are secreted in the parotid glands of the toad to protect the animal from predators [2,3].

In mammals, MBG is part of the *endogenous digitalis-like factor (EDLF)* family. This group of endogenous compounds is reportedly involved in sodium homeostasis and act as endogenous cardiac inotropes with vasoconstrictive and cardiotonic properties [4,5]. The main action of MBG is the inhibition of the α1-isoform of Na^+, K^+-ATPase (α1-NKA). In the kidney α1-NKA is the main sodium pump isoform present in the epithelium of the proximal tubule and thick ascending limb of Henle's loop [6–8]; α1-NKA is also ubiquitously distributed in other ion-transporting epithelia such as in vascular smooth muscle cells [9–11]. In humans, elevated levels of MBG are associated with hypertension and natriuresis [12–15]. With α1-NKA also acting as a signal transducer, MBG can also modulate other cellular functions such as endothelial cell growth and differentiation, apoptosis, and collagen production [6,8,16,17]. Growing evidence is pointing that, in response

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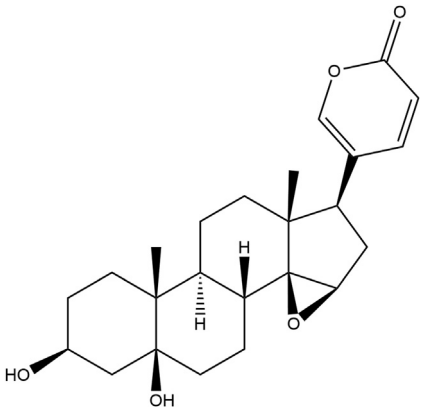

Fig. 1. Marinobufagenin is composed of a steroid core structure with a 6-membered lactone ring.

to volume expansion and/or sodium overload, the upregulation of Angiotensin II, in turn, promotes MBG synthesis from cholesterol in the placenta [18] and/or in the adrenal cortex [19,20] via the bile acidic pathway [21].

Fluid retention-induced production of MBG has first been observed in dogs [22] and in salt-sensitive hypertension in Dahl Sensitive (DS) rats [23]. More recently, elevated levels of endogenous MBG-like immunoreactive material has been reported in patients with volume expansion-mediated hypertensive states such as essential hypertension [12,15], congestive heart failure [5,24], chronic renal failure [25,26], uremic cardiomyopathy [27], and preeclampsia (PE) [6,28–30]. In patients with preeclampsia, elevations in arterial pressure have been associated with increased plasma levels of MBG-like material [28,29]. In non-pathological pregnancy, moderate elevations of MBG-like compound were correlated to the absence of hypertension [29]. The usual extracellular fluid volume increase during pregnancy would be expected to stimulate the secretion of a putative natriuretic hormone, like MBG, to regulate the extracellular fluid volume [6,23,31].

A more direct role for MBG in preeclampsia etiology has also been suggested as MBG is shown to impair cytotrophoblast invasion of the uterine wall, which is critical for an efficient placental development and pregnancy maintenance [32,33].

With a growing body of literature implicating MBG in the pathogenesis of preeclampsia, the potential of MBG as a predictive biomarker for preeclampsia has been proposed [6,34,35]. The determination of MBG plasma levels early in pregnancy may possess not only great predictive value, but also raises the possibility for early intervention, e.g., by administration of resibufogenin, an MBG antagonist [34,36], and hence may result in the prevention of preeclampsia. However, the potential of MBG as a useful biomarker for preeclampsia remains to be fully established [34].

Currently, only marinobufagenin-like material has been determined in biological fluids using two different immunoassays [13,37]. Immunoassays are known to be prone to cross-reactivity issues, often leading to poor specificity, and tend to exhibit high variability at low concentrations [38,39]. Application of the two aforementioned in two studies for MBG plasma levels in preeclampsia (PE) compared to normal pregnancy (NP) led to discordant MBG levels: Lopatin et al. [29] reported for NP = 250 ± 27 pg/mL and PE = 1052 ± 40 pg/mL whereas Agunanne et al. [28] reported for NP = 12 ± 2 pg/mL and PE = 59 ± 17 pg/mL [2]. Based on these very divergent MBG levels reported, it is not unthinkable that cross-reactivity with e.g., another EDLF compound with the MBG-directed antibodies can occur and therefore lead to inaccurate determinations of MBG-like material concentrations.

What is more, one can even question whether MBG can be detected in maternal blood regardless. For another endogenous cardiotonic steroid, namely ouabain, also from the EDLF family, it has recently been proven that no endogenous ouabain is detectable in human plasma when using a highly specific, antibody-free analytic method, i.e, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [40]. Therefore confirmation of the detectability of endogenous MBG in maternal blood should be re-assessed, and this by a highly specific method which avoids the generation of false positive MBG data.

In the clinical biology field, LC-MS/MS methods are increasingly being used as they exhibit numerous advantages compared to immunoassays: this technology is characterized by excellent specificity, sensitivity and the ability to multiplex analytes [41]. Contrary to immunoassays, LC-MS/MS enables the unambiguous identification of analytes: this follows the availability of a set of assay parameters, like retention times, precursor mass, product ions; which jointly allow for the unequivocal identification of an analyte of interest.

The only LC-MS study on MBG plasma levels reported in the literature has been published in 2005 [42]. Based on recent guidelines for LC-MS analysis and technological progress made in the field [43], the described method seems to fail by a low sensitivity, the use of a non-deuterated internal standard and only one poor-specific mass transition to authenticate MBG. Further investigations using a greater number of more specific mass transitions are thus needed to confirm the identity of endogenous MBG with a high degree of certainty.

In the current paper, we report on the development of a highly specific qualitative LC-MS/MS assay for MBG. Then we investigated whether the presence of MBG could be confirmed in the plasma of pregnant women obtained in the 2nd trimester of their pregnancies,

This study is the first one, as far as we know, to authenticate MBG in human plasma, and more specifically in pregnancy, by use of a highly specific analytical method that relies on unique mass transitions combined to the use of a deuterated internal standard.

2. Materials and methods

2.1. Chemicals

MBG was obtained by an in-house developed extraction method from the *Bufo marinus* crystallized toad venom (Alpha-Biotoxine, Belgium). After a purification procedure, the purity of MBG was determined by different spectral techniques including Thin Layer Chromatography (TLC)-UV and TLC-MS. d_3–25-hydroxyvitamin D3 (d3-25-ohd), used as the internal standard (ISTD) for the MS/MS method development, was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Fig. 2). HPLC/MS-grade solvents and salts for the LC mobile phase were purchased from Fischer Optima (Dublin, Ireland). EDTA plasma samples from 20 pregnant women (median age: 23 (IQR 29–38)) collected in the 2nd semester of their pregnancy (average gestational week of collection 15.25 (SD:1.52 weeks)) were purchased from BBI solutions (Cardiff, UK).

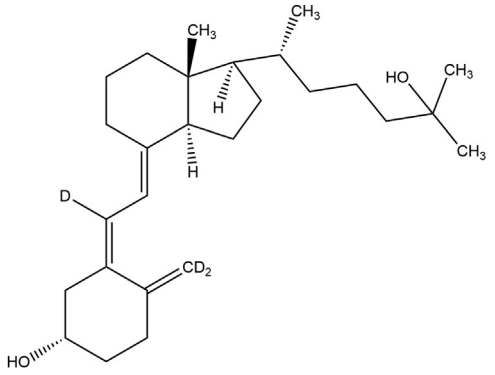

Fig. 2. d_3-25-hydroxy-vitamin D_3 structure.

2.2. Apparatus

The Agilent (Santa Clara, CA, USA) LC-MS equipment for the qualitative analyses of plasma samples included an Infinity 1200 series HPLC system and a 6460 triple-quadrupole mass spectrometer equipped with the JetStream® technology. The software used was MassHunter® Version B.06.00 (2012). Mobile phase A consisted of 10 mM NH4OAc pH 4.5 in 97:3 (v/v) H2O:MeOH and mobile phase B consisted of 10 mM NH4OAc pH 4.5 in 50:45:5 (v/v/v) MeOH:IPA:H2O. Column used was an Agilent Pursuit 3 PFP column (100 mm × 3 mm 3 µm) preceded by a guard column PFP. A 10 min elution gradient was used as followed for the separation of plasmatic components: 0% of mobile phase B from 0 to 1.5 min, gradually increased to 100% B at 3 min, and maintained isocratic for 3 min, returned at 0% B at 7.5 min and maintained isocratic at 0% B until 10 min (for column re-equilibration). The injection volume was 7.5 µL and the mobile phase flow rate was set at 0.45 mL/min. For the quality control (QC) and BBI samples analyses, an Agilent PFP Pursuit guard column (10 × 3 mm 3 µm) was added in front of the analytical column. The samples were kept at 4 °C in the autosampler. Mass spectral ionization, fragmentation, and acquisition conditions were optimized on the tandem quadrupole mass spectrometer by using electrospray ionization (ESI) in the positive mode. To enable authentication of MBG in human plasma, the mass spectrometer was operated in the dynamic multiple reaction monitoring (dMRM) mode with 7 mass transitions for MBG and 2 mass transitions for the internal standard (IS), d3-25-ohd (Table 1). Data were collected and processed by use of MassHunter software (version B.06.00).

2.3. Preparation of working solutions and quality control (QC) material

To set-up the qualitative LC-MS/MS assay and assess the MS method parameters, a MBG working solution of 250 pg/mL solubilized in methanol was prepared from a stock solution at 1 mg/mL MBG. The same solution was used to assess the repeatability of the assay. The stock solution was stored and stable at − 20 °C; stability was repeatedly assessed for 1 year. No MBG signal intensity decrease was observed (data not shown). QC samples are composed of pooled plasma from all 20 BBI patients' samples enriched with MBG. The QC samples were daily prepared for each batch analysis. For batches analyses, QC samples were prepared at 3 different concentration levels for MBG: high QC corresponds to the pooled BBI sample fortified with MBG; fortification corresponded to a 150 ng/mL addition. The middle QC is constituted of the unfortified pooled sample; and the low QC which agreed with a 10-fold dilution of the unfortified pooled sample. Dilution was done with 0.01 M saline phosphate buffer (PBS; Sigma®) + 0.5% w/v Bovine Serum Albumin (Sigma®).

Table 1
LC-MS/MS detection settings for MBG and d_3-25-ohd: 7 mass transitions with their own collision energy were recorded for MBG whereas 2 mass transitions were used for the internal standard.

Compound	t_R (min)	Transition ($m/z \rightarrow m/z$)	Fragmentor (V)	Collision Energy (eV)
Marinobufagenin	5.235	401 → 145	132	35
		401 → 159	132	30
		401 → 243	132	25
		401 → 339	132	16
		401 → 347	132	20
		401 → 365	132	15
		401 → 401	132	0
d_3–25-hydroxyvitamin D3	5.547	386.3 → 108	98	53
		386.3 → 159	98	37

2.4. Sample preparation

Regarding the sample preparation procedure for the QC and BBI plasma samples kept at − 80 °C, a reliable, automatable and cost-efficient extraction method was applied. 40 µL of plasma were thawed on ice at room temperature and vortexed for 10 s. Each aliquot was spiked with 10 µL of ISTD and vortexed at 1000 rpm for 10 min at 4 °C. The protein precipitation solvent with a proprietary formulation containing MeOH:IPA:200 mM NH_4OAc supplemented with 3,5-Di-tert-4-butyl-hydroxytoluene was retrieved from − 20 °C and stirred for 1 min. 335 µL of protein precipitation solvent was added to each sample and vortexed at 1400 rpm at 4 °C for 10 min followed by a sonication for 2 min. Sample was then maintained at − 20 °C for 30 min and then centrifuged at 18 kg for 15 min at 4 °C (Sigma 3–16KL® refrigerated centrifuge). 300 µL of supernatant was transferred to high recovery injection vials (in glass) and concentrated to dryness at 40 °C for 1 h (Refrigerated Centrivap Concentrator, Labconco®). Reconstitution was elaborated in 50 µL of mobile phase A, vortexed for 10 s and 7.5 µL transferred to the LC-MS platform. All samples were prepared on the day of analysis and kept in the autosampler at 4 °C.

2.5. Pregnant women plasma samples

The method was applied to plasma samples from 20 healthy pregnant women at around 15 weeks of pregnancy extracted with the sample preparation method. The samples were run twice (2 separate extractions on 2 different days). As the setup of the quantitation method was at that stage still in progress, MBG wasn't fully quantified in these samples. However, a ratio MBG/ISTD composed of MBG transition 401→365 m/z peak area on ISTD transition 386 → 159 m/z peak area could be calculated. The ratio allows to evaluate the quantity of MBG in each sample corrected for sample preparation and instrumental variations by the internal standard area ratio.

3. Results and discussion

3.1. Protein precipitation-based extraction method

Prior to the set-up of an optimized dosage method for MBG plasma levels, an effective extraction method is needed to clean and concentrate the plasma samples. Sample clean-up is necessary for improved specificity and sensitivity and allows for avoiding ion suppression due to sample matrix components in MS analyses. Several extraction techniques have been considered and protein precipitation (PPT) was preferred due to its high efficiency, straightforward handling, affordable price, and wide variety of extracted compounds. A gentle thawing procedure of plasma samples on ice for 20 min at room temperature was preferred to avoid matrix components degradation. Sonication after the protein precipitation and freezing of the precipitated sample were performed to allow the separation of protein aggregates. Given the qualitative aspect of our methodology, no extraction recovery was calculated.

3.2. Set up of the LC-MS/MS assay

LC–MS/MS is a powerful tool that has seen extensive growing interest into routine bioanalysis and clinical practice [38,39,44]. In LC-MS/MS, separation of analytes is performed with liquid chromatography and detection is based on the specific mass to charge ratio (m/z) of each molecule. The use of the triple quadrupole technology allows discrimination of closely similar analytes based on their fragmentation pattern and a significant decrease in background noise. Compared to immunoassays, LC-MS/MS advantages are numerous but the most significant are: improved specificity and discrimination for low molecular weight analytes, high-throughput with automatization. In addition, it can multiplex methods allowing the simultaneous analysis of multiple

compounds in a single short time LC–MS/MS run for a wide dynamic range.

Even with monoclonal antibody techniques, it is difficult to raise antibodies that recognize a single precise molecular structure. Additionally, the acquisition and/or manufacturing process of anti-MBG antibodies remains a complex and time-consuming process. On the contrary, LC-MS/MS is easier and faster to develop in-house and presents lower running costs [38,45].

The improved specificity for LC-MS/MS is obtained thanks to the use of Single or Multiple Reaction Monitoring (SRM or MRM). In MRM detection mode, the first quadrupole is set at the specific *m/z* ratio of the solute of interest, the parent mass, and only ions with these *m/z* ratios pass through and are fragmented in the collision cell. In the third quadrupole, only characteristic fragment ions thereof are selectively measured [46,47]. The combination of the three quadrupoles are producing a single analyte-specific ion-pair for which the detection sensitivity is vastly increased. This specific ion-pair is called a mass transition. The enhanced specificity allows for improved accuracy at lower concentration compared to immunoassays, which are often in poor agreement with each other for the same analyte [38]. Moreover, recently developed mass spectrometer devices are able to reach very low limits of quantification (LOQ, in the range of the pg/mL).

Use of positive electrospray ionization enabled effective ionization and sensitive detection of MBG and d3-25-ohd. Cone voltages were adjusted for further enhancement of the sensitivity by improving the electrospray ionization. The collision energy (CE) was adjusted for each fragment ion. The CE that was producing the highest signal intensity for each mass transition was chosen (see Table 1).

Fig. 3 shows the product-scan spectrum of the molecular and fragment ions of MBG standard stock solution at 250 pg/mL. To allow correct identification of MBG, seven mass transitions, resulting from combining parent mass to fragment ion mass, were selected for MBG. The fragment ion with highest intensity was 365 *m/z* and corresponds to a loss of two water molecules. As the loss of one or two water molecules is not considered as specific enough, fragment ion 347 *m/z* corresponding to a loss of three water molecules was chosen to characterize MBG. Fragment ion 349 *m/z* was selected as well thanks to its selective character as it is unique to MBG. Two mass transitions for d_3-25-ohd were selected. The fragment ion at 159 *m/z* with highest intensity, yet selective, and a more selective one at 108 *m/z* were chosen to allow differentiation between isobaric compounds.

One of the disadvantages of LC-MS/MS is signal suppression or enhancement due to sample matrix components. The use of stable isotope-labeled internal standards (ISTD) in MRM analyses is crucial to compensate for that matrix effects and helps to avoid potential variations in sample extraction and injection volume as it undergoes the same analysis protocol as the compound of interest [39]. In standard LC-MS/MS dosage methods, the isotopically labeled identical of the analyte of interest should ideally be used as ISTD. However, this was not possible for MBG because of commercial unavailability and because of the absence of suitable synthetic procedures for in-house fabrication. The ISTD d3-25-ohd was selected thanks to its structural and mass likeness with MBG. Even if the retention time is close to MBG, both compounds could be differentiated by use of specific mass transitions for MBG compared to d3-25-ohd.

Based on immunometric-assessed endogenous MBG plasma levels in preeclamptic patients (ranging from 60 to 1000 pg/mL), the reference MBG solution used to assess MS parameters was chosen to contain 250 pg/mL. As the quantitation method was at that stage not fully developed nor validated, no LOD or LOQ could be determined. Yet, the repeated analyses of the MBG standard solution at 250 pg/mL (n = 10) demonstrated a high intensity and a good repeatability (CV% = 0.16% for retention time and CV% = 14.1% for signal intensity). The peak showed high resolution from the noise suggesting the ability of the method to quantify low levels of MBG within acceptable limits (see Fig. 4).

3.3. Analyses of human pregnant women plasma samples

Results of MBG investigation in the BBI study pregnant women plasma samples are listed in Table 2 (only samples that are positive for MBG are shown). Among the 20 different samples, MBG could only be identified in 3 pregnant women. The ratios of MBG/ISTD in the 3 concerned women tend to decrease over the experiments on the 2 different days. This phenomenon is not likely to be due to extraction procedure or instrumental variations as the content in ISTD stays similar over the 2 days, but it might be explained by a difference in the thawing procedure of plasma samples. Although this reduction in MBG content, the same patient samples show MBG over the 2 experiments. For each of these positive samples, all mass transitions, and especially the most selective ones, could be observed at MBG retention time (5.230 min). A non-pregnant control sample has been done but data are not showed. Chromatograms of the 7 mass transitions for MBG and 2 mass transitions for ISTD in a 17 weeks' pregnant woman and in high

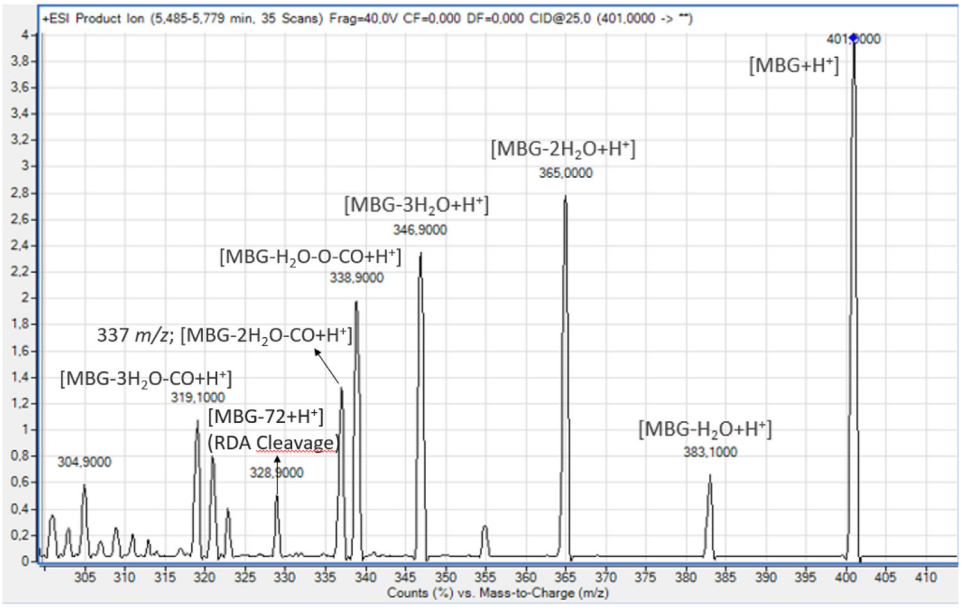

Fig. 3. Product ion mass spectrum of the pure MBG standard solution at 250 pg/mL: The parent ion at 401 *m/z* and the main daughter ions of MBG groups are clearly visible and identified: 383 *m/z*, 365 *m/z*, 347 *m/z* corresponding to successive losses of water molecules; 339 *m/z*, 337 *m/z* and 319 *m/z* corresponding to successive losses of water and carbonyl and finally 329 *m/z* corresponding to the RDA cleavage (Retro Diels Alder Reaction) at the C-ring of the steroid skeleton of MBG.

Fig. 4. Chromatograms of the MBG mass transitions 401 → 339 *m/z* (***a***, upper part) and 401 → 347 *m/z* (***b***, lower part) of MBG stock solution at 250 pg/mL; retention time (Tr) for MBG is 4.922 min.

Table 2
Results of the MBG/ISTD quantifier transition (401→ 347 *m/z* and 386 → 159 *m/z* respectively) peak area ratio in BBI study pregnant women plasma samples.

BBI Sample	Age	Gestational age	Ratio MBG/ ISTD area (Day 1)	Ratio MBG/ ISTD area (Day 2)
Woman 1	34 years old	17 weeks	1.4976	0.2402
Woman 2	26 years old	16 weeks	1.5401	0.1263
Woman 3	29 years old	14 weeks	0.1733	0.3623

QC are shown in Fig. 5.

When comparing chromatograms obtained with both the high QC solution at 150 ng/mL and the pregnant woman sample, preliminary observations can be made. As a peak was clearly differentiated at a retention time that was identical for high QC and pregnant woman plasma sample, for every single mass transition, we can confirm the authentication of endogenous MBG in pregnant women plasma.

The retention time-shift between the chromatogram obtained with the MBG stock solution at 250 pg/mL and those obtained from pregnant woman plasma samples is explained by the addition of the guard column in front of the analytical column. When analyzing samples in complex matrices such as plasma, a guard column is added to reduce the matrix effect and to avoid the clogging up of the ion source. The guard column allows also to increase on a significant way the lifetime of the analytical column.

These pioneer exclusive results confirm observations made in pregnancy with previously described MBG immunometric methods [28,29,48,49]. As we didn't have access at that time to non-pregnant women plasma samples we couldn't conclude anything about the rise of MBG plasma levels in case of pregnancy. However, these analyses are now in progress.

Despite the lack of identification of MBG in every pregnant woman plasma sample, the authentication of MBG by LC-MS/MS in plasma samples coming from 3 different pregnant women could allow us to confirm the occurrence of endogenous human MBG. The absence of a distinctive peak for MBG in the other 17 samples could be explained by the intra-individual variations of endogenous MBG plasma levels at such low level of concentration.

Until now, only one research team did investigate MBG in human plasma by use of LC-MS/MS but their relied on the use of only one single poor-specific mass transition (401 → 383 *m/z*). This mass transition, corresponding to the loss of one molecule of water, is the less specific one as it appears to be common for 25-hydroxyvitamin D3 [50]. Moreover, the authors used Proscillaridin A, another bufadienolide compound from plant origin as internal standard [42]. However, the choice of that substance as internal standard is likely not relevant as the description of proscillaridin A-immunoreactive material was unable to certify the absence of endogenous proscillaridin A [51,52].

Thanks to the enhanced selectivity of the two consecutive mass filters combined to the chromatographic separation, this novel highly specific LC-MS/MS method allows the identification of MBG in plasma at levels lower than ng/mL. Previously described immunoassays could only identify MBG-like material without any certitude about the occurrence of MBG in human plasma. The antibodies used in both published immunoassays were characterized by cross-reactivity phenomena preventing the authentication of MBG identity in plasma [2]. This novel approach gives the clinicians a promising opportunity to further assess the use of MBG for early preeclampsia risk assessment in pregnant women and in other volume expansion-mediated hypertensive disorders.

4. Conclusion and perspectives

We developed a dedicated MBG LC-MS method, able to detect and identify MBG in pregnant women plasma samples. Thanks to the high specificity of the distinct 7 mass transitions used in this LC-MS/MS assay, we confirmed the presence of endogenous marinobufagenin in pregnant women. The promising results suggest privileging MBG detection via such instrumental technique eliminating confusion with former marinobufagenin-like material.

The utility of LC-MS/MS in the detection and monitoring of biomarkers for disease processes as diverse as the pregnancy-specific syndrome of preeclampsia demonstrates its need as well as its potential. From now on, the setup of a dedicated LC-MS quantification assay for MBG plasma levels recording combined to other biomarkers in non

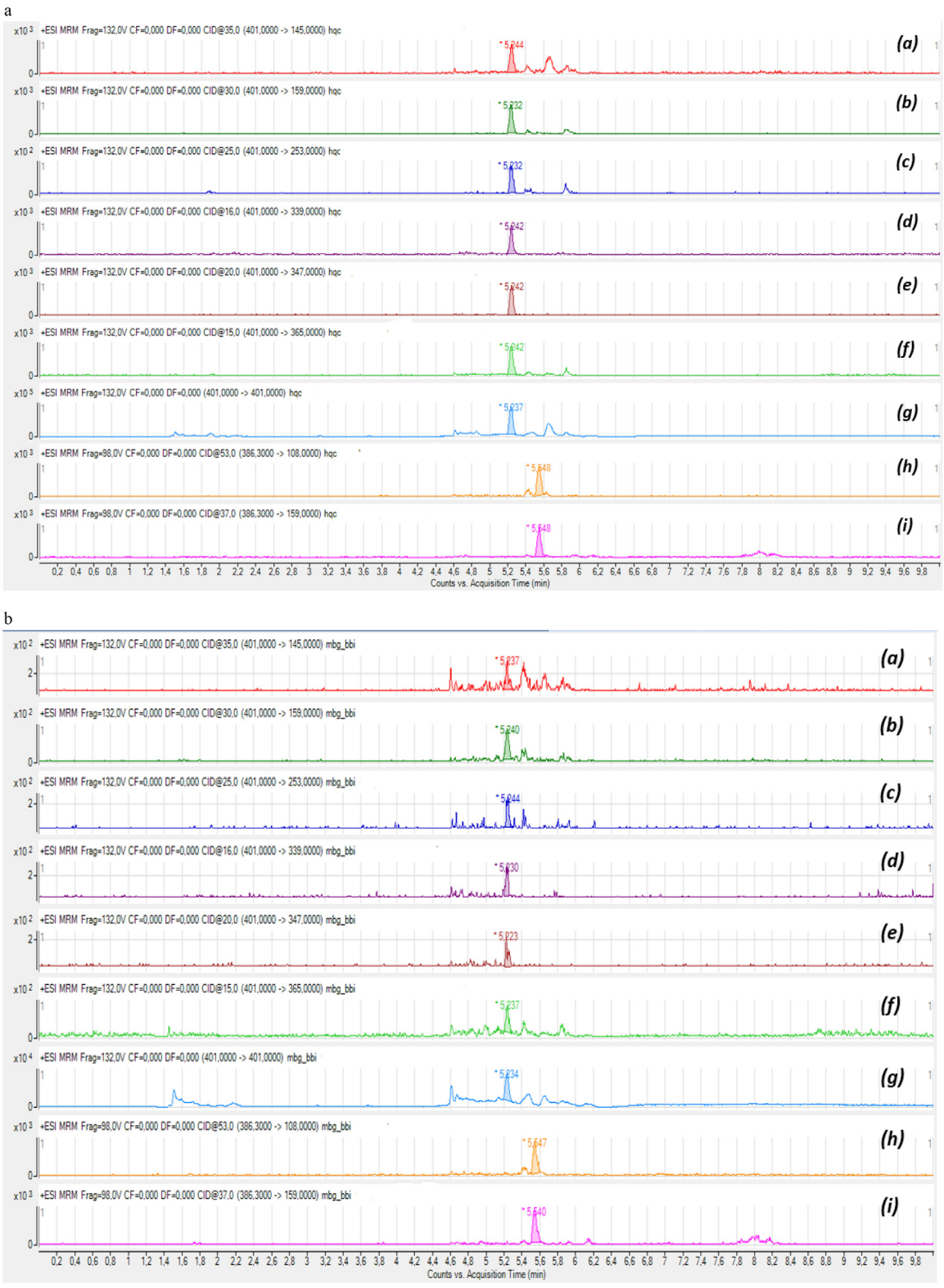

Fig. 5. a Chromatogram of the 7 mass transitions of MBG (***a-g***) and the 2 mass transitions of ISTD (***h-i***) in the high concentrated quality control sample: The retention time of MBG is defined at 5.24 min (use of the guard column) and ISTD elutes very close with a retention time at 5.54 min **b** Chromatogram of the 7 mass transitions of MBG (***a-g***) and the 2 mass transitions of ISTD (***h-i***) in a plasma sample issued from a 17 weeks pregnant woman: Despite the presence of noise in the background, we could make out a peak et the retention time for MBG (tr MBG = 5.24 min) for each of the specific mass transition as for the d_3–25-hydroxyvitamin D3 (d_3-25-ohd) (tr ISTD = 5.54 min). These pioneering results obtained in 2 other pregnant women (data not showed) allow us to authenticate, for the first time, endogenous MBG.

pathological pregnancy compared to preeclampsia will allow us to elucidate the discrepancy concerning MBG plasma levels obtained with previously published immunoassays. The high versatility of that kind of assay will allow us to extent the study of MBG role to other volume expansion-hypertensive states paving the way for novel potential therapeutic agents.

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