Identification of marinobufagenin in plasma as a promising LC-MS assay for preeclampsia risk assessment

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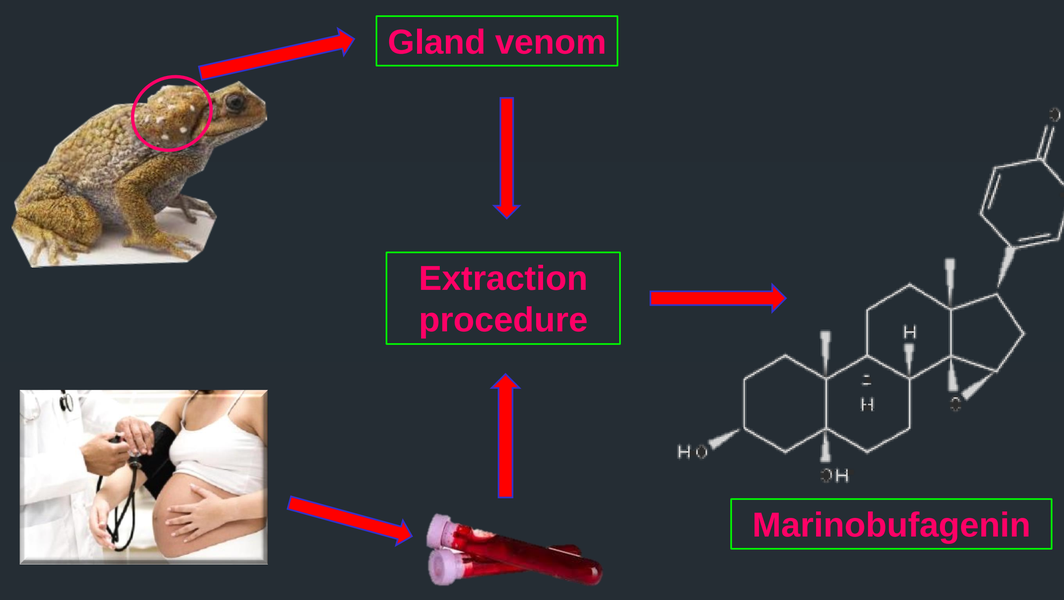
Introduction

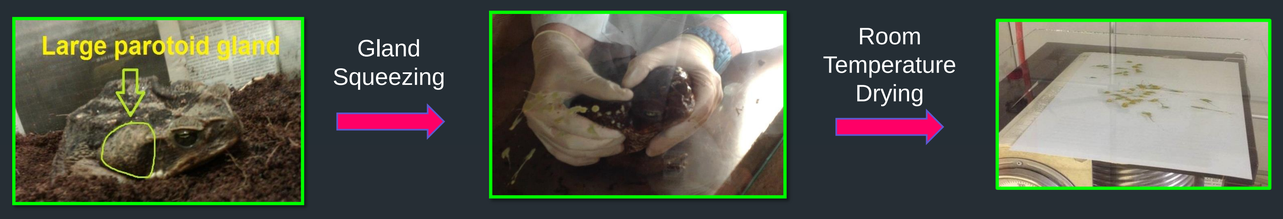
- Marinobufagenin (MBG), a cardiotonic bufadienolide, is a selective inhibitor of the α_1 subunit of Na^+,K^+-ATPase. Bufadienolides are mainly located in the parotoid gland secretions of some toad species but can also be found in mammals.
- Due to its vasoconstrictive, cardiotonic and natriuretic activities, endogenous MBG is implicated in volume expansion-mediated hypertensive states such as preeclampsia.
- Increased plasma MBG has been observed in preeclamptic women and a rat model for preeclampsia (PE) [1-3]. The increased MBG production appears prior to the development of the symptoms, leading us to consider MBG as a biomarker for PE.
- This hypothesis involves an accurate and sensitive analytical method for MBG plasma levels quantification in order to further investigate the implications of MBG in PE. Currently, only marinobufagenin-like material has been found in humans. Here we report the identification of MBG in non-pregnant human plasma as well as in a plasma sample obtained from a 15 weeks pregnant woman utilising a LC-MS assay, opening the perspective of investigating the potential of MBG in preeclampsia risk assessment.

Ref: [1] Vu, H.V., et al., *American Journal of Nephrology*, 2005. **25**(5): p. 520-528. [2] Agunanne, E., et al., *Amer J Perinatol*, 2011. **28**(EFirst): p. 509-514. [3] Lopatin, D.A., et al., *Journal of Hypertension*, 1999. **17**(8): p. 1179-1187. [4] Abi-Ghanem, D., et al., *Journal of Immunoassay and Immunochemistry*, 2011. 32(1): p. 31-46. [5] Fedorova, O.V., et al., *Circulation*, 2002. 105(9): p. 1122-1127.



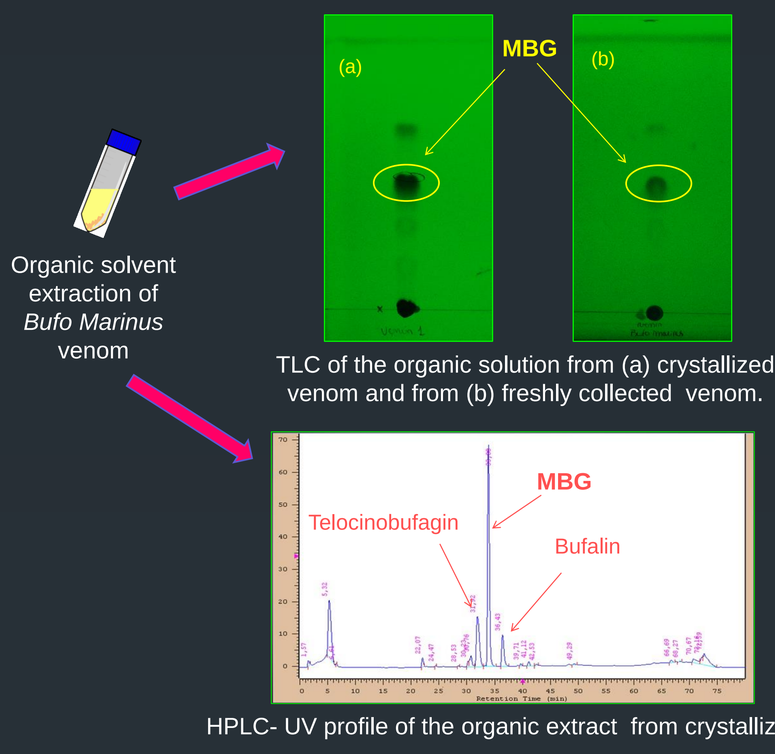
Extraction of pure MBG

1) Venom Collection



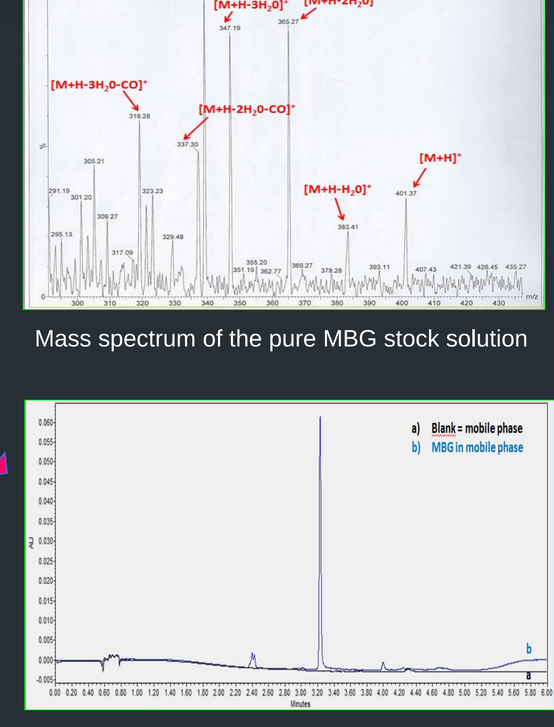
- Toad parotoid glands secretion are known to contain bufadienolide compounds. MBG is the major cardiotonic steroid in the *Bufo Marinus* venom.
- Given that no MBG standard is commercially available, we needed to develop an effective extraction and purification method to acquire the reference compound.
- To this end we considered both freshly collected and crystallized *Bufo Marinus* venom; and then optimized a method to extract MBG from toad venom. The identity of MBG has been confirmed by UPLC-UV and mass spectrometry.

2) Extraction and identification of MBG in *Bufo Marinus* venom



TLC of the organic solution from (a) crystallized venom and from (b) freshly collected venom.

HPLC- UV profile of the organic extract from crystallized venom



Mass spectrum of the pure MBG stock solution

UPLC-UV chromatogram of the MBG stock solution

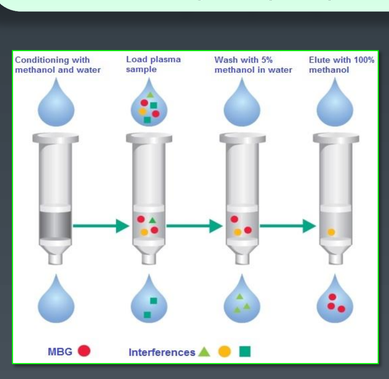
HPLC-UV and MS conditions	
Stationary Phase	Waters® Atlantis dC18
λ UV-detection	296 nm
Mobile Phase	Gradient elution; A: acidified H_2O, B: ACN
Flow	1 mL/min
MS Ion source and mode	Electrospray; positive ion mode
MBG	$[M+H]^+$: m/z 401

UPLC-UV conditions	
Stationary Phase	Waters® BEH C18
λ UV-detection	296 nm
Mobile Phase	Gradient elution; A: acidified H_2O, B: ACN
Flow	0.4 mL/min

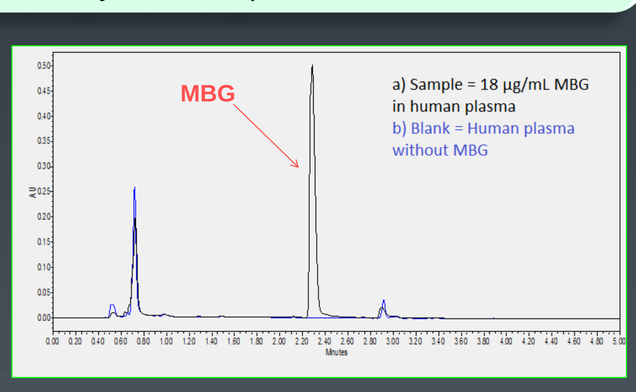
MBG caracterization in human plasma

1) Solid Phase Extraction (SPE) process

The setup of a sensitive quantification method for MBG plasma levels starts with an extraction from plasma samples by SPE. This preliminar step is essential for sample clean up and concentration. Several SPE sorbent phases were tested: clean up with Waters® HLB (hydrophilic lipophilic balanced) cartridge gave the best extraction yield (92%) for a relatively clean sample.



Standard SPE procedure

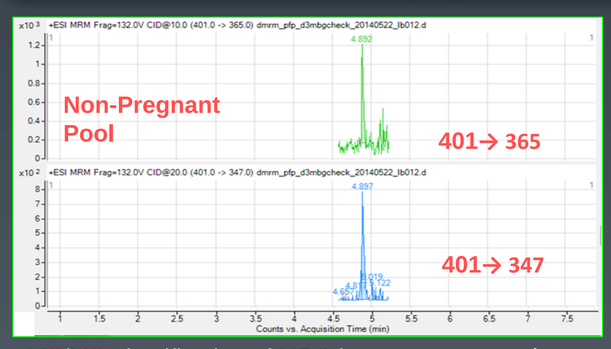


UPLC Chromatogram after extraction of MBG from enriched human plasma with HLB cartridge

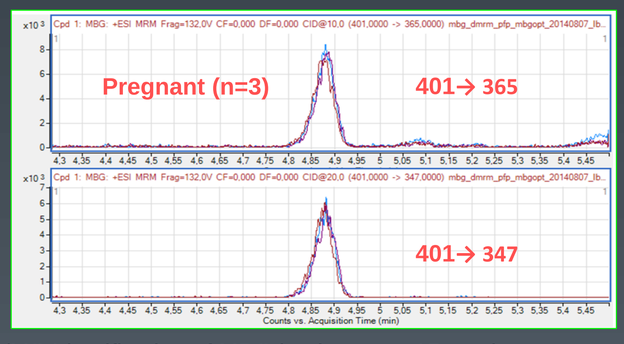
2) MS/MS caracterization in human plasma: developed in Metabolomic Diagnostics

Currently, only MBG-like material has been determined in human samples using two different immunoassays, but for both the results were distorted due to cross-reactivity [4,5]. Using the purified MBG, a sensitive MRM based LC-MS/MS assay was developed for MBG. Preliminar tests showed that MBG could be easily detected at 0,25 ng/mL. The LC-MS/MS assay allowed us to detect endogenous MBG in both plasma obtained from healthy non pregnant volunteers and from a 15 weeks pregnant woman.

HPLC-MS/MS conditions	
Stationary Phase	Agilent® PFP
Mobile Phase	Organic/aquous gradient elution
Flow	0.45 mL/min
MBG	$[M+H]^+$: m/z 401
Ion source and mode	Electrospray, positive ion mode



MS/MS Identification of MBG in non-pregnant plasma



MS/MS Identification of MBG in plasma at 15 weeks pregnancy

Conclusion

- We obtained pure MBG as a standard for analytical method development following extraction of MBG from *Bufo Marinus* crystallized venom and subsequent purfiication
- A SPE sample clean-up step for MBG from human plasma has been developed with an extraction yield of 92%.
- A sensitive LC-MS/MS assay was developed which allowed us to authenticate MBG in human plasma: MBG could be identified in non-pregnant healthy patients as well as in early pregnant (15 weeks) volunteers. For so far we know, these initial findings are the first to highlight MBG by LC-MS/MS as thus far only MBG-like compounds have been reported for human plasma..
- With MBG plasma levels being reported as increased when preeclampsia manifestates , this dosage method once fully developed will help to quantify MBG plasma levels in early pregnancy, giving the clinicians a promising opportunity to assess the potential of MBG for early preeclampsia risk assessment. In addition the availability of an LC-MS/MS method will help the elucidation of some research questions such as: the biosynthetic origin of MBG and/or what role MBG exactly plays in the PE syndrome.

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