Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2016 May 1; 1020: 90–95. doi:10.1016/j.jchromb.2016.03.023.

Screening for toxic phorbol esters in jerky pet treat products using LC–MS

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Abstract

Since 2007, the U.S. FDA's Center for Veterinary Medicine (CVM) has been investigating reports of pets becoming ill after consuming jerky pet treats. Jerky used in pet treats contains glycerin, which can be made from vegetable oil or as a byproduct of biodiesel production. Because some biodiesel is produced using oil from *Jatropha curcas*, a plant that contains toxic compounds including phorbol esters, CVM developed a liquid chromatography-mass spectrometry (LC–MS) screening method to evaluate investigational jerky samples for the presence of these toxins. Results indicated that the samples analyzed with the new method did not contain *Jatropha* toxins at or above the lowest concentration tested.

Keywords

Jatropha curcas; Phorbol esters; Jerky pet treats; High resolution; Accurate mass; LC–MS method

1. Introduction

Since 2007, FDA's Center for Veterinary Medicine (CVM) has received numerous reports of pet illnesses following the consumption of jerky pet treats [1]. Reports involve treats from multiple manufacturers and include a range of brands and flavors; these treats typically contain chicken or duck breast meat, glycerin, and spices as their main ingredients. Efforts by numerous laboratories to determine the underlying cause of the treat-associated illnesses have been unsuccessful to date and have focused on screening jerky treats for various contaminants and toxic agents. Investigations have included microbiological, compositional, and chemical toxico-logical testing as well as studies to determine whether irradiation of

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jerky treats leads to the formation of potentially toxic compounds. Particular attention has been given to glycerin as a potential source of toxicants.

Jerky pet treats are generally made from poultry (chicken, duck, turkey, etc.) breast meat that has been soaked in glycerin and dried in an oven. Glycerin used in jerky treats may be produced from vegetable oil or as a byproduct of biodiesel manufacturing. One source of biodiesel fuel is oil from the seeds of *Jatropha curcas*, a toxic, drought-hardy plant prevalent in Latin American, Asian, and African countries [2]. If ingested, these plants can cause severe gastrointestinal irritation, dehydration, and death in humans and animals [3–5].

The toxicity of *J. curcas* is ascribed mainly to a toxic protein, curcin, and a group of diterpene esters termed phorbol esters, which are present in relatively high concentrations in the seeds of some *J. curcas* varieties [2]. While heat denatures curcin and renders it nontoxic, it does not affect phorbol esters [6]. Six *J. curcas* phorbol esters, named *Jatropha* factors (JFs) C1–C6, have been isolated and characterized previously (Fig. 1A–F) [2,7]. All six compounds have the same molecular formula and are intramolecular diesters of the diterpene, 12-deoxy-16-hydroxyphorbol (Fig. 1G). Upon mechanical extraction of oil from *Jatropha* seeds, the majority of JFs (~70%) remain in the oil fraction [8]. There is a concern, therefore, that these compounds are a potential cause of toxicity in jerky treats made using contaminated glycerin.

We developed an LC–MS method to determine if *Jatropha* toxins were present in jerky pet treats. Because standards of JFs were not commercially available, we used phorbol 12-myristate 13-acetate (PMA, Fig. 1H), a phorbol ester analogue, for preliminary method development work. The method was based on an initial solvent extraction followed by a clean-up using hydrophilic lipophilic polymeric solid phase extraction cartridges. The esters were chromatographically resolved and analyzed by mass spectrometry. The method yielded satisfactory recoveries of the JFs, and was suitable for the analysis of investigational samples. We used this new method to analyze several investigational jerky pet treat samples.

2. Materials and methods

2.1. Materials

HPLC grade methanol and acetonitrile were purchased from Burdick & Jackson (Muskegon, MI, USA). A Milli-Q Reference Ultra-pure Water System (Millipore Corp., Billerica, MA, USA) was used to obtain deionized water. Formic acid (95%) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Food-grade glycerin (99.7%) was purchased from US Pharmacopeia (Rockville, MD, USA). Oasis HLB SPE cartridges were purchased from Waters Corp. (Milford, MA, USA). Potassium phosphate buffer (0.3 M, pH 7) was prepared using analytical grade potassium dihydrogen phosphate and potassium monohydrogen phosphate purchased from Fisher Scientific (Fair Lawn, NJ, USA). Whatman 60 A, 70–230 mesh silica gel and silica gel 60 F254 2.5 × 7.5 cm were used for flash chromatography and thin layer chromatography, respectively.

J. curcas oil (25 mL) was obtained from F. Beckford, University of Florida, IFAS Extension through the CVM's Veterinary Laboratory Investigation & Response Network (Vet-LIRN).

Vet-LIRN also provided investigational samples obtained from consumer cases and retail markets. Samples were stored at -15°C on receipt. Pet jerky treat samples to be used as controls were prepared in our laboratory using store-bought fresh chicken breast.

2.2. Extraction of jatropha factors (JFs) from *J. curcas* oil

We extracted JFs from oil (25 mL) of *J. curcas* using previously described methods [8]. The *Jatropha* oil was extracted with methanol (25 mL) using a magnetic stirrer (300 rpm) at 60°C for 5 min. The resulting mixture was gravity separated. After three extractions, the methanol layers were combined and evaporated under nitrogen and freeze dried to obtain ~3 g of residue. The residue was dissolved in dichloromethane (DCM), loaded onto a silica gel column, and eluted with 1, 3, and 5% methanol in DCM. The fractions were monitored by thin layer chromatography under short wavelength UV. The JFs eluted with 3% methanol in DCM, which was evaporated to yield a crude standard enriched with JFs (~16 μg).

2.3. Preparation of control jerky treat samples

Control jerky treat samples were made using chicken breast tenderloins bought in a grocery store. The tenderloins were soaked in food-grade glycerin for 20 min before drying. Tenderloins were placed on the dehydrator rack for 30 min (Sedona Dehydrator; Tribest Co., Anaheim, CA, USA) after soaking to allow excess glycerin to drip onto a paper towel placed under the rack. Samples were then dried at 68°C for 48 h, left on the rack for 2 h to cool, and placed in labeled whirl pak bags. Prepared samples were stored at room temperature.

2.4. Sample extraction procedure

A schematic presentation of the sample preparation is given in Fig. 2. A jerky treat sample (50 g) was homogenized using a Robot Coupe blender (Robot Coupe USA Inc., Ridgeland, MS, USA) for 5–10 min until it was ground to a fine powder-like consistency. The ground sample was stored at -15°C until analysis.

The ground sample was thawed and weighed (0.5 g) into a 15 mL polypropylene centrifuge tube. Potassium phosphate buffer (0.3 M, 0.5 mL) and methanol (1.5 mL) were added and the sample was sonicated for 20 min. After centrifugation (Centra GP8R centrifuge, International Equipment Co., Chattanooga, TN, USA) at 4000 rpm for 10 min, the supernatant was carefully transferred to a 50 mL centrifuge tube. The sample pellet was re-extracted by adding methanol (2 mL) and vortexing on a laboratory shaker (VWR VX-2500 multi tube vortex mixer, Radnor, PA, USA) for 20 min. After centrifugation at 4000 rpm for 10 min, the supernatant was transferred to the 50 mL tube containing the first extract. The combined extract was diluted to 20 mL with water and vortex-mixed. An aliquot (10 mL) was used for the automated solid phase extraction (SPE) procedure using the RapidTrace SPE Workstation (Caliper Life Sciences, Alameda, CA, USA).

Oasis HLB SPE cartridge was conditioned with methanol (2 mL) and water (2 mL). Sample was loaded onto the SPE cartridge at a rate of 0.4 mL/min. The cartridge was dried by passing nitrogen through it at approximately 30 psi for 3 min. The cartridge was rinsed with 2 mL of 20% methanol and dried again for 3 min. Analyte elution was accomplished with methanol (4 mL) at 0.5 mL/min into a 15 mL centrifuge tube. Eluate was evaporated to

about 200 μ L on a TurboVap LV evaporator (Zymark Corporation, Hopkinton, MA, USA) at 40 °C under a steady flow of nitrogen (12 psi) and was reconstituted up to 500 μ L with a 65:35 water: methanol solution. Sample was then filtered through a 0.20 μ m PVDF Whatman syringe filter into an autosampler vial. An injection volume of 10 μ L was used for LC–MS analysis.

2.5. Daily operation and quality control procedure

Control jerky treat sample was used to prepare blanks and fortified samples. Known amounts of the JFs-enriched fraction were added to the control jerky to give fortification levels of 50 ng/g and 300 ng/g (as PMA equivalent). For analysis of unknown samples, these fortified control jerky treat samples were used as QC material. Seven solvent calibration standards in the range of 10–750 ng/mL were prepared using a 10 μ g/mL PMA working standard. Each day, three injections of 100 ng/mL solvent calibration standard were made to equilibrate the LC–MS system and to establish system suitability. This was followed by the analytical run consisting of a solvent blank, two matrix blanks, calibration standards from low to high, a solvent blank, up to ten unknown samples in duplicate, QCs, and a solvent blank followed by the reanalysis of the calibration standards. Both Q-Exactive and AB Sciex mass spectrometers were used for the analysis.

2.6. Instrumentation

Q-Exactive high resolution mass spectrometer (MS) (Thermo Fisher Scientific, Bremen, Germany) coupled to an Accela 1250 LC (Thermo Fisher Scientific, Bremen, Germany), a 4000 triple quadrupole MS (AB Sciex Instruments, Foster City, CA) coupled to a Shimadzu Prominence LC (Shimadzu, Columbia, MD, USA), and an Agilent (Santa Clara, CA, USA) quadrupole time-of-flight (Q-TOF) 6530 MS coupled to an Agilent 1290 Infinity LC were used for analysis. All LCs were equipped with a refrigerated autosampler and a binary pump. The autosampler tray was set at 15 °C.

2.6.1. LC separation—A C8 column (2.0 mm $\times$ 150 mm, 3 μ m, 100 Å, Phenomenex) maintained at 40 °C was used. Mobile phase A and B were 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile, respectively. Injection volume was 10 μ L. In Q-Exactive and Sciex systems, mobile phase was delivered at a combined flow rate of 450 μ L/min under a gradient program. The gradient started at 65% mobile phase B and ramped to 100% by 13 min, and was held for 4.5 min. The solvent system was ramped back to initial conditions over 0.5 min and held for 12 min before starting the next injection. In the Q-TOF system, a flow rate of 400 μ L/min was used. The gradient was ramped from 70% mobile phase B to 100% in 10 min, held for 5 min, and ramped back to initial conditions over 1 min.

2.6.2. Mass spectrometric detection—Q-Exactive MS was operated in positive ionization mode using HESI source at 350 °C. Accurate mass measurement (< 2 ppm) was ensured through calibration with the manufacturer's calibration mixture consisting of caffeine, the tetrapeptide, MRFA, and Ultra-mark. Full scan experiment from m/z 200–750 in positive mode was acquired at a resolving power of 70,000 FWHM. A spray voltage of 4 kV, capillary temperature of 350 °C, and heater temperature of 300 °C were used. Sheath gas flow and auxiliary gas flow were 40 and 10 units, respectively. Molecular ion at m/z

711.38910 and three fragment ions at m/z 311.16420, m/z 675.36790, m/z 693.37850 were monitored. Detection was based on calculated exact mass and on the retention time of the target ions. Data were evaluated using Xcalibur 2.2 software.

AB Sciex 4000 MS was used with an electrospray interface. Source conditions were as follow: source temperature 500 °C, spray voltage 5.5 kV, gas 1 (GS1) 50 units, gas 2 (GS2) 50 units, curtain gas flow 30 units, and collision gas 10 units. Nitrogen was used as the nebulizer and collision gas. Analysis was done in multiple reaction monitoring (MRM) mode. The protonated molecule of JFs was not observed under the mass spectrometric conditions. Four prominent ion transitions, 693 → 311, 675 → 293, 311 → 265, 311 → 165, were used for MRM mode. The declustering potential was set at 70 V. Other acquisition parameters are given in Table 1. Data acquisition and analysis were performed using Analyst software (version 1.5.1). Integrations of the reconstructed ion chromatograms consisting of the diagnostic product ions were automatically carried out by Analyst processing method and were manually evaluated.

Q-TOF was used with electrospray ionization with Jet Stream Technology. The parameters used were: fragmentor, 165 V; nozzle, 100 V; Vcap, 3500 V; nebulizer, 50 psi; N₂ drying gas, 12 mL/min, 325 °C; N₂ sheath gas, 5 mL/min, 200 °C. The MS was calibrated daily according to the manufacturer's recommendations. The instrument was operated in the high-resolution (4 GHz), lower mass range (< m/z 1700) positive ion mode. Data were collected in both centroid and profile formats. Reference masses at m/z 121.05087 (purine) and m/z 922.00980 (hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine) were continually introduced along with the LC stream for accurate mass calibration. Data acquisition and analysis were performed using MassHunter software (Agilent). Integrations of the chromatograms were automated and manually evaluated.

3. Results and discussion

As part of FDA's investigation into reports of pet illnesses following jerky treat consumption, we developed an LC–MS analytical method that would enable rapid screening of jerky treats for *Jatropha* toxins. Using this method we analyzed several investigational samples. The method produced recoveries acceptable for the intended purpose; recoveries were 51% and 69% for control jerky treat matrix fortified with JF-enriched fraction at 50 ng/g and 300 ng/g (as PMA equivalent), respectively. The calibration curve was linear in the range of 10–750 ng/mL with a coefficient of determination (R^2) exceeding 0.99.

Standards of JFs were not commercially available, so an analogous phorbol ester, PMA, was used for the initial method development work. Q-Exactive and AB Sciex mass spectrometers were used for the analysis. In the meantime, another group worked to develop a standard by extracting and isolating JFs from seed oil of *J. curcas* grown in an area known to have high content of JFs. Q-TOF instrument was used for this analysis.

To ensure that the control did not contain JFs, we prepared our own jerky treat controls using fresh chicken breast and food grade glycerin (US Pharmacopeia). The developed method involves an initial solvent extraction followed by a clean-up of the extract using solid phase

extraction cartridges. The jerky matrix is highly complex, and the matrix components could potentially interfere with the analyte determination. The SPE clean-up in the method removes most of these matrix components reducing matrix interference significantly. An external standard calibration curve of peak area of PMA versus concentration was constructed using linear regression without any weighting, and excluding the origin. It included both bracketing standard curves, and was applied to all samples within the bracket. Reports were generated with the integrated peak areas, calculated concentrations, and retention time.

The initial solvent extract of the jerky matrix had a pH of ~4–5. During our initial method development we found that low pH of extract was detrimental to the recovery of JFs. Since JFs are unstable compounds [2], the low recoveries were likely due to degradation of JFs under acidic extraction conditions. Recoveries increased once we adjusted the sample's pH to 7 by adding a phosphate buffer before extraction with solvent.

Recovery of JFs from the jerky matrix was determined by using the ratio of the chromatographic peak area count of the analyte fortified before extraction to the peak area count of the analyte in the sample fortified post-extraction representing 100% recovery.

Though a rigorous validation procedure was not conducted, daily quality control was maintained for each batch by including system suitability samples, negative controls (matrix blanks), positive controls (fortified matrix), solvent blanks, and a calibration curve. Calibration samples were run at the beginning and end of each batch bracketing the investigational samples.

We used high resolution mass spectrometry to obtain accurate masses of ions, which enabled us to use our crude standard of *Jatropha* toxins in place of a certified standard. This could not have been accomplished by the use of a low resolution mass spectrometer with a less selective nominal mass. JFs in the enriched fraction were characterized by exact mass measurement with less than 2 ppm mass accuracy of the protonated molecular ion, sodium adduct ion, and several fragment ions using Q-TOF mass spectrometer. A series of peaks appeared (Fig. 3A) in a narrow time window with an m/z of 711.3883 (Fig. 3B), corresponding presumably to JFs as reported previously [2]. The measured m/z closely matches the calculated value of 711.3891 for the protonated molecular ion for the formula, $C_{44}H_{55}O_8$, with less than 2 ppm mass accuracy. Additionally, previously reported fragment ions [6,9] were observed at the same retention time as the protonated molecular ion (Fig. 3B); they are m/z 693.3778, 675.3683, 311.1641, 293.1520 and 263.2350 with mass accuracies <2 ppm. The detection of these ions with high mass accuracy and at the same retention time as the parent molecular ion indicates the presence of JFs in this JFs-enriched fraction. Furthermore, the formula generation algorithm of Agilent Mass Hunter software generated the correct formula, $C_{44}H_{54}O_8$, with a high score of 97 and a mass accuracy of 1.1 ppm. Additional support for identifications came from Q-Exactive accurate mass data with a higher resolution (70,000) than was obtained with the Q-TOF MS, and the MRM experiments with the triple quadrupole MS as described later in the discussion.

MRM experiments were carried out on a triple quadrupole instrument. JFs share a common basic fragment of the 12-dehydroxy, 16-hydroxyphorbol nucleus (Fig. 1G) with m/z 311. Most phorbol esters, including PMA, share this nucleus [6]. In lieu of the JFs standard, we followed the strategy of Ichihashi et al. [6], who used this common ion to optimize MRM parameters. By means of the flow injection method, we introduced PMA and optimized parameters for the fragment ion at m/z 311. We then used these values for the MRM experiments of the JFs.

Ichihashi et al. [6] observed a weak protonated molecular ion but strong fragment ions at both low and high cone voltages, and proposed that the fragment ions were produced by in-source decay. Our observations were consistent with their work; the protonated molecular ion at m/z 711 was insignificant in JFs to be used as a precursor ion. Therefore, we selected prominent daughter ions at m/z 693, 311, and 675 as the precursor ions for MRM.

Previously, JFs have been quantified relative to PMA since JFs were not available as standards [6]. We followed a similar strategy to semi-quantitate JFs in the extract relative to PMA. We used two transitions common to PMA and JFS, $311 \rightarrow 165$ and $311 \rightarrow 265$, in the multiple reaction monitoring (MRM) mode in the triple quadrupole MS for the quantitation.

We tested ten investigational jerky treat samples, and found no JFs present above 50 $\mu\text{g/g}$ (as PMA equivalent), the lowest concentration tested. Fig. 4A shows chromatograms of a control matrix (Fig. 4A1), a control matrix fortified with JFs (Fig. 4A2), and an investigational sample (Fig. 4A3) in Q-Exactive MS, and corresponding chromatograms (Fig. 4B1–B3) in AB Sciex MS. In the Q-Exactive MS, the traces in each set correspond to extracted ions m/z 711.3891, 693.3785, 675.3679, and 311.1642 from top to bottom. In the Sciex MS, the traces correspond to transitions $311 \rightarrow 165$, $675 \rightarrow 293$, and $693 \rightarrow 311$. The control sample and the investigational sample in Q-Exactive MS do not show the four ions characteristic of the JFs. Similarly, the characteristic transitions of the JFs are also absent in the control and investigational samples in the MRM experiments.

4. Conclusion

In order to support an FDA investigation into reports of pet illnesses following the consumption of jerky pet treats, we developed a liquid chromatography-mass spectrometry (LC–MS) screening method to evaluate investigational jerky samples for the presence of *Jatropha* factors toxins. The results of the study demonstrated that the investigational samples tested did not contain *Jatropha* toxins at or above the lowest concentration tested.

Acknowledgments

The authors thank F. Beckford, University of Florida, Institute of Food and Agricultural Extension, and Y. Shen, Office of Regulatory Affairs, FDA for providing *J. curcus* oil and/or seeds. The authors also thank Mike Filigenzi, Linda Aston, and Robert Poppenga of California Animal Health and Food Safety, UC Davis School of Veterinary Medicine for their discussions and collaboration with CVM to explore potential methods for identifying *Jatropha* factors in jerky samples.

Funding

This project was supported in part by an appointment to the Research Participation Program at the Center for Veterinary Medicine administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

References

- [1]. FDA. [accessed 10.11.15] Jerky Pet Treats. 2016. <http://www.fda.gov/animalveterinary/safetyhealth/productsafetyinformation/ucm360951.htm>
- [2]. Haas W, Sterk H, Mittelbach M. Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. J. Nat. Prod. 2002; 65:1434–1440. [PubMed: 12398539]
- [3]. Wink, M.; Koschmieder, C.; Sauerwein, M.; Sporer, F. Biofuel and Industrial Products from *Jatropha Curcas*. Dbv-Verlag Graz University of Technology; Graz, Austria: 1997. Phorbol esters of *Jatropha curcas*-biological activities and potential applications; p. 160-166.
- [4]. Makkar, HPS.; Becker, K. Toxic Plants and Other Natural Toxicants. CAB International; Wallingford, UK: 1997. *Jatropha curcas* toxicity: identification of toxic principle(s); p. 554-558.
- [5]. Devappa RK, Makkar HPS, Becker K. *Jatropha* toxicity—a review. J. Toxicol. Environ. Health Part B. 2010; 13(6):476–507.
- [6]. Ichihashi K, Yuki D, Kurokawa H, Igarashi A, Yajima T, Fujiwara M, Maeno K, Sekiguchi S, Iwata M, Nishino H. Dynamic analysis of phorbol esters in the manufacturing process of fatty acid methyl esters from *Jatropha curcas* seed oil. J. Am. Oil Chem. Soc. 2011; 88:851–861.
- [7]. Hirota M, Suttajit M, Suguri H, Endo Y, Shudo K, Wongchai V, Hecker E, Fujiki H. A new tumor promoter from the seed oil of *Jatropha curcas* L., an intramolecular diester of 12-deoxy-16-hydroxyphorbol. Cancer Res. 1988; 48:5800–5804. [PubMed: 3167837]
- [8]. Roach JS, Devappa RK, Makkar HPS, Becker K. Isolation stability and bioactivity of *Jatropha curcas* phorbol esters. Fitoterapia. 2012; 83(3):586–592. [PubMed: 22245089]
- [9]. Baldini M, Ferfua C, Bortolomeazzi R, Verardo G, Pascali J, Piasentier E, Franceschi L. Determination of phorbol esters in seeds and leaves of *Jatropha curcas* and in animal tissue by high-performance liquid chromatography tandem mass spectrometry. Ind. Crop Prod. 2014; 59:268–276.

Fig. 1. Structures of *Jatropha* factors C1–C6 (1A–1F), 12-deoxy-16-hydroxyphorbol (1G) and phorbol 12-myristate 13-acetate (1H).

Fig. 2.
Schematic presentation of the sample preparation of jerky treats.

Fig. 3. JFs enriched fraction in Agilent Q-TOF: (A) Overlaid extracted ion chromatograms of the characteristic ions of JFs, (B) Mass spectrum showing characteristic ions at m/z 711 (protonated molecular ion), 693, 675, 311, 293, and 263.

Fig. 4. Chromatograms of a control matrix (A1), a control matrix fortified with JFs (A2), and an investigational sample (A3) in Q-Exactive MS, and corresponding chromatograms (B1–B3) in AB Sciex MS.

Table 1

Collision energies (CE) and exit potentials (CXP) of the multiple reaction monitoring transitions of JFs in positive ion mode in AB SCIEX 4000 triple quadrupole MS.

Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	CE	CXP
693	311	50	12
675	293	50	12
311	265	30	15
311	165	80	14