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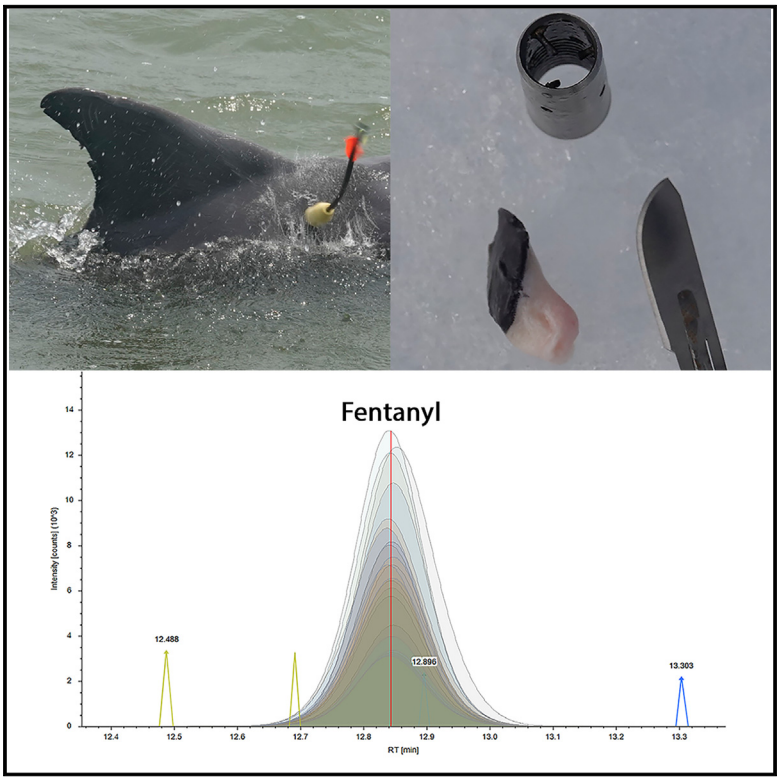
Pharmaceuticals in the blubber of live free-swimming common bottlenose dolphins (*Tursiops truncatus*)

Graphical abstract



Highlights

- Bottlenose dolphins are bioindicator species of ecosystem health
- Pharmaceuticals found in the blubber of 30 dolphins (24 live) in the Gulf of Mexico
- Detected pharmaceuticals included opioids, muscle relaxants, and sedatives
- Pharmaceuticals in the marine ecosystem appear to be a long-standing issue

Authors

Anya Isabelle Ocampos, Makayla A. Guinn, Justin Elliott, Christiana Wittmaack, Carrie Sinclair, Hussain Abdulla, Dara N. Orbach

Correspondence

dnorbach@gmail.com

In brief

Environmental science; Ecology; Zoology

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Pharmaceuticals in the blubber of live free-swimming common bottlenose dolphins (*Tursiops truncatus*)

Anya Isabelle Ocampos,[1] Makayla A. Guinn,[1] Justin Elliott,[1] Christiana Wittmaack,[2] Carrie Sinclair,[3] Hussain Abdulla,[1] and Dara N. Orbach[1,4,*]

[1]College of Science, Texas A&M University-Corpus Christi, Corpus Christi TX 78412, USA
[2]Precision Toxicological Consultancy, Monroe NC 28110, USA
[3]National Oceanic and Atmospheric Administration, Southeast Fisheries Science Center, Mississippi Laboratories, Pascagoula MS 39567, USA
[4]Lead contact
*Correspondence: dnorbach@gmail.com
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SUMMARY

Pharmaceuticals prevent and treat diseases, yet inappropriate intake can result in harmful effects including mortality. Contaminants have become recurrent public and wildlife health concerns. Bioaccumulation of contaminants can occur throughout trophic levels of the food web. Dolphins are apex predators often used as sentinel species to assess the health of marine ecosystems because their lipid-rich blubber stores contaminants. We used blubber samples collected from live free-swimming and postmortem common bottlenose dolphins (*Tursiops truncatus*) in the Gulf of Mexico to explore the presence of pharmaceutical contaminants in the marine ecosystem. Targeted analysis of blubber using ultra-performance liquid chromatography coupled with Orbitrap Fusion Tribrid mass spectrometry confirmed the presence of fentanyl, carisoprodol, or meprobamate in 30 of the 89 dolphins assessed. We provide the first detection of human pharmaceuticals stored in live free-swimming marine mammals, with important implications for understanding ecosystem health.

INTRODUCTION

Pharmaceutical drugs are therapeutic substances used in human and veterinary medicine to diagnose, treat, cure, or prevent disease(s). Improper use of pharmaceuticals can cause harmful effects including antibiotic resistance, addiction, overdose, and mortality.[1,2] For example, improper use of veterinary pharmaceuticals in terrestrial mammals may result in hypersensitivity reactions, gastric effects, hepatotoxicity, anaphylaxis, and mortality.[3] Pharmaceuticals and active pharmaceutical ingredients (APIs) have become emerging micropollutants and are a growing global concern.[4,5] The presence of pharmaceutical residues and APIs has been reported in freshwater ecosystems, rivers, and oceans worldwide.[6,7] The transfer of human pharmaceuticals into aquatic environments often occurs through insufficient treatment of wastewater effluent and untreated discharge from pharmaceutical manufacturing facilities.[7–10] Conventional treatment methods implemented by wastewater treatment plants have pharmaceutical removal efficiencies ranging from 23% to 54%.[11] Residual veterinary pharmaceuticals in animal manure may enter aquatic systems through runoff.[12] Dietary medicinal doses are commonly directly lost from aquaculture and shrimp farming to aquatic ecosystems.[8,13]

Pharmaceuticals are biologically active compounds that interact with specific physiological pathways. Pharmaceuticals including hypocholesterolemic drugs, beta blockers, cardiovascular drugs, non-steroidal anti-inflammatory drugs, steroid hormone drugs, psychiatric drugs, and antibiotics can bioaccumulate in marine invertebrates (e.g., crustaceans, mussels, and shrimp) and fish[14–16]; most studies on trophic transfer in aquatic environments were conducted in freshwater rivers or lakes lacking higher aquatic apex predators.[7,15,17,18] The exposure of fish to anxiolytic (oxazepam) and analgesic (carbamazepine, paracetamol)/angiotensin II receptor blocker (irbesartan)/nonsteroidal anti-inflammatory (naproxen, diclofenac) drugs can result in behavioral alterations and endocrine disruptions, respectively.[19–21] Humans can indirectly consume many classes of pharmaceuticals from drinking water and food products[22] (e.g., contaminated fish and seafood), which may be a public health concern.[23] Drug residue intake from consuming edible animal tissues exposed to antimicrobial drugs (e.g., sulfamides and lincosamides) may have short-term (e.g., allergic reactions) and long-term (e.g., mutagenic, carcinogenic, and teratogenic) effects on human health.[24] Reports of human pharmaceuticals stored in free-swimming apex marine predators are limited to a few studies.[25–27] One population of bull sharks (*Carcharhinus leucas*) inhabiting a wastewater-impacted river had detectable human contraceptive and anti-depressant metabolites in plasma.[26] Recently, muscle relaxants or migraine medications were detected in low concentrations (<1 ng/g) in the

livers and antihistamines in the blubber of seven postmortem dolphins, highlighting that detection is dependent on the tissue matrix and properties of the medications (e.g., lipophilic).[27] Antidepressants were reported in the feces of captive killer whales (*Orcinus orca*), although it is not clear if the animals were treated with the pharmaceuticals, and there was no evidence of drug storage.[28]

Common bottlenose dolphins (*Tursiops truncatus*; hereafter "dolphins") are susceptible to bioaccumulation of lipophilic compounds from pollutants and are effective bioindicators of ecosystem health in contaminant research; common bottlenose dolphins have lipid-rich blubber that can store contaminants and be sampled relatively minimally invasively, high trophic level positions in food webs, long lifespans, and relatively fast metabolisms.[29–31] It remains unclear what are the chronic effects of pharmaceuticals in dolphins. We used liquid chromatography coupled with Orbitrap Fusion Tribrid mass spectrometry to evaluate the presence and concentrations of human pharmaceuticals stored in the blubber of free-swimming, *in vivo*, and postmortem dolphins off three locations in the Gulf of Mexico (GoM). Level 1 confidence was achieved. Other matrices known to accumulate pharmaceuticals in vertebrates were not sampled as that would require the subjects to be postmortem (e.g., liver, brain, and kidney tissues) or entail highly invasive and logistically constraining collection techniques (e.g., plasma and urine) that would substantially reduce the sample size.

Fentanyl (an opioid analgesic for severe pain that is 100× more potent than morphine[32]), carisoprodol (a muscle relaxant for painful musculoskeletal injuries), and meprobamate (a sedative and anxiolytic drug for treating anxiety disorders) were selected based on an untargeted analysis (Methods S1). Fentanyl targets the brain and binds to opioid receptors. Upon ingestion, fentanyl passes through the stomach and intestine where it can subsequently be defecated or transferred to the liver and metabolized by CYP3A4 (CYP3A29 is the homolog in cetacean skin[33]) and excreted in urine. Inhalation and dermal contact with fentanyl reach the brain, fat, kidney, and liver by blood transport. The half-life of fentanyl is 3–7 h.[34] Carisoprodol and meprobamate are metabolized in the liver by the cytochrome P450 system.[35,36] The half-life of carisoprodol is 2 h, while its metabolite meprobamate has a half-life of 10 h.[37] The metabolism of fentanyl, carisoprodol, and meprobamate has not been assessed in cetaceans to our knowledge.

RESULTS

LOD and LOQ

A total of 89 blubber samples were analyzed from different dolphins (83 biopsy collected, 6 postmortem) inhabiting Redfish Bay, TX (RB, n = 46), Upper Laguna Madre, TX (ULM, n = 13), and Mississippi Sound, MS (MS, n = 30) in the GoM (Figure 1). Of these 89 tissue samples, 63% were collected from male (n = 56) and 37% from female dolphins (n = 33). Samples from postmortem animals were collected from five dolphins around Corpus Christi Bay, TX (adjacent to RB), and one dolphin in Baffin Bay, TX (near the connection to ULM). The limit of detection (LOD) for fentanyl, carisoprodol, and meprobamate was 0.07, 0.30, and 2.00 ng/mL (ppb), respectively. The limit of quantification (LOQ) for fentanyl, carisoprodol, and meprobamate was 0.30, 0.50, and 10 ng/mL, respectively.

Pharmaceutical detection

The identification of pharmaceutical compounds was confirmed based on the Orbitrap MS1 data of their adducts ($[M + H]^+$ and $[M + Na]^+$) with a mass error of less than 5 ppm, along with isotope pattern matching and a retention time deviation within ±0.4 min of the corresponding standards. The pharmaceutical compounds were detected in the blubber of 30 dolphins (Table 1). For further details, please refer to the standard preparation and instrumental analysis sections. However, quantification was unattainable as no samples reached LOQ for any analyte. Fentanyl was found in 18 dolphins sampled by remote biopsy across the study sites and in all six postmortem dolphins (27% of blubber samples; Table 1; Figures 2, S1, and S2). Carisoprodol was detected in five dolphins (5.6% of blubber samples) while meprobamate was detected in one dolphin (1.1% of blubber samples; Table 1; Figures S1 and S2). One targeted pharmaceutical was detected in each of the samples where detections occurred.

Sex and temporal patterns

The distribution of pharmaceuticals between the sexes mirrored the demographics, with 63% of detections from males and 37% from females. RB had a high detection of pharmaceuticals relative to biopsy samples collected (RB: 49% biopsy samples, 62% pharmaceutical detections; ULM: 15% biopsy samples, 17% pharmaceutical detections; MS: 36% biopsy samples, 21% pharmaceuticals detected). Pharmaceuticals were found in 12 historic blubber samples from MS (2013) comprising 40% of total pharmaceutical detections.

DISCUSSION

The data support that narcotic (opiate) analgesics and skeletal muscle relaxants can reach apex marine predators. The low (non-quantifiable) concentrations of opiate analgesics and muscle relaxants in dolphins in both Texas and Mississippi waters reinforce the need for large-scale assessments across trophic levels, water columns, and ecosystems globally; such widespread investigations will help ascertain the severity and source(s) of contamination.[25] We recommend initial efforts in areas with dense human populations and prominent aquaculture/fishing industries where humans may be most at risk. As 40% of all detected pharmaceuticals (n = 12) were found in the historical samples, pharmaceutical pollution may be a long-standing issue that has been largely overlooked; assessment of historic water and tissue samples across marine taxa for pharmaceutical detection will provide insights into the duration of the issue.

Bioaccumulation of fentanyl through the consumption of contaminated prey cannot be discredited as the log Kow is 4.05.[38] Bioaccumulation of carisoprodol is unlikely as the estimated log Kow is 2.36.[39] Meprobamate does not bioaccumulate as the log Kow is 0.07.[40] It is also possible that the dolphins were recently exposed to the pharmaceuticals. Monitoring concentrations of all three pharmaceuticals within the water column may help pinpoint sources of exposure. Fentanyl, carisoprodol, and

Figure 1. Study sites within the Gulf of Mexico with boating survey routes outlined in red

Remote biopsy samples were collected in Redfish Bay, TX, from 2012 to 2014 and 2022, in Upper Laguna Madre, TX, during 2022, and in Mississippi Sound, MS, during 2013. Postmortem dolphin tissue samples were collected from the Redfish Bay and Upper Laguna Madre vicinities in 2022–2023.

meprobamate can cross the placental barrier with varying effects of toxicity.[41,42] Offloading of fentanyl, carisoprodol, and meprobamate through lactational transfer occurs in some mammalian species,[43,44] although this has not yet been explored in cetaceans.

Detection of narcotic (opiate) analgesics and skeletal muscle relaxants was overall higher in dolphins inhabiting RB and ULM compared to MS. In the years immediately following the 2010 *Deepwater Horizon* oil spill (during which biopsy surveys were conducted), dolphins inhabiting Mississippi but not Texas experienced increased stranding rates.[45] However, dolphins inhabiting South Texas were deemed priority stocks by the National Oceanic and Atmospheric Administration due to High Cumulative Threat scores from compounded exposure to oil and gas pollution, vessel traffic, dredging and construction, and algal blooms.[46] Chronic exposure to multiple stressors can compromise the immune integrity of cetaceans, rendering them particularly susceptible to infection, reproductive failure, and mortality.[47] There is a need to proactively monitor contaminants of emerging concern (CECs) to inform mitigation efforts, particularly in regions with chronic and diverse stressors to marine biota.

Assessing the potential source(s) of exposure of the dolphins to narcotic (opiate) analgesics and skeletal muscle relaxants was beyond the scope of this study. However, our untargeted analysis was conducted on a dolphin found within one year of the largest liquid fentanyl drug bust in United States history in the adjacent county,[48] and fentanyl is stable in the ocean. The presence of human pharmaceuticals in free-swimming marine species is not usually assessed, and when tested, a targeted approach is often used due to the impracticality of testing all pharmaceutical classes.[20,49] Initial non-targeted analyses of contaminants, especially in apex marine predators, allow for a broad assessment of the health of marine environments. Non-targeted studies may aid regulatory authorities in identifying and prioritizing which CECs to monitor and mitigate. However,

Table 1. Number of dolphins with detected pharmaceuticals in blubber samples across three study sites

Pharmaceutical	Redfish Bay, TX	Upper Laguna Madre, TX	Mississippi Sound, MS
L	41 (9:32)	12 (3:9)	30 (18:12)
PM	5 (3:2)	1 (0:1)	–
Fentanyl			
L	12 (3:9)	4 (1:3)	2 (2:0)
PM	5 (3:2)	1 (0:1)	–
Carisoprodol			
L	2 (0:2)	–	3 (2:1)
PM	–	–	–
Meprobamate			
L	1 (0:1)	–	–
PM	–	–	–

The top numbers in each cell denote samples collected by remote biopsy of live (L) dolphins, and the bottom numbers denote samples collected from postmortem (PM) dolphins. The ratio of females to males is presented in brackets. Remote biopsy samples were collected in Redfish Bay from 2012 to 2014 and 2022, in Upper Laguna Madre during 2022, and in Mississippi Sound during 2013. Postmortem dolphin tissue samples were collected from the Redfish Bay and Upper Laguna Madre vicinities in 2022–2023.

non-targeted approaches should be followed by targeted analyses that verify CEC presence and quantify concentrations to enhance the understanding of marine ecosystem health.

The detection of fentanyl in substantially more blubber samples than carisoprodol and meprobamate is expected as fentanyl readily distributes to fat. As carisoprodol and meprobamate have low bioconcentration factors in lipids, their detection in blubber (but not in blank samples) underscores the research potential for advanced mass spectrometry. It is unlikely (although potentially possible) that the detected analytes were in plasma within blubber as we did not use bloody biopsy samples. Concentrations below LD_{50} suggest that acute toxicity is not of concern. However, chronic exposure and cumulative effects are unknown in marine mammals. Sublethal effects from chronic exposure to some pharmaceuticals can occur in fish, crustaceans, and arthropods.[50] Fentanyl was detected in all postmortem dolphins in this study, and orphenadrine, pizotifen, or promethazine were detected in 70% of postmortem dolphins in a different study,[27] possibly indicating some comorbidities. While toxicological studies on marine mammals are limited due to conservation concerns and policies, *in vitro* and *in silico* studies may enable risk assessments of pharmaceuticals and other pollutants[51] and assessments of synergistic effects.

Limitations of the study

No sample reached LOQ for any analyte. Without quantification of pharmaceutical concentrations, the risk assessment to the dolphins and ecosystem is curtailed. However, the presence of carisoprodol and meprobamate in any blubber sample despite their low accumulation in lipids and brief half-lives in mammals underscores the value of detection. Future research that quantifies pharmaceutical concentrations will be of value. A mass spectrometer capable of detecting concentration as low as femtomolar levels might enable the quantification of the low concentrations that occur in dolphins. Despite matches in retention time and m/z, we cannot definitively exclude the possibility of coeluting isobaric or isomeric metabolites. However, we are confident in our quality control and verification process as we had high mass accuracy combined with retention time, used labeled standards, repeatedly tested blanks, and used MS2 matching with our in-house database.

Remote biopsy sampling of blubber provides a way to assess thresholds of survival in marine mammals before possible comorbidities and cascading ecological effects occur. Blubber samples were used as a biological matrix because blubber can be collected from live free-swimming dolphins by remote biopsy. Remote biopsy is non-lethal and substantially less invasive, costly, and logistically prohibitive compared to the live animal captures needed to obtain plasma and urine or postmortem animals needed to obtain liver, kidney, and brain tissues. We did

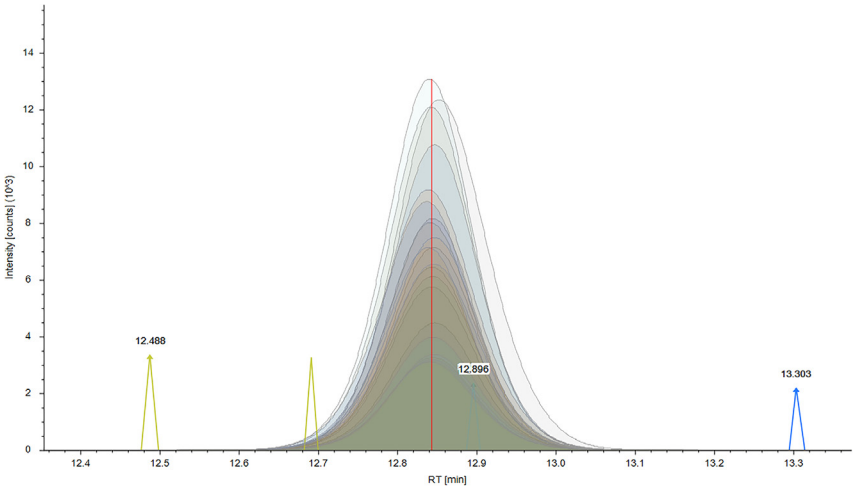

Figure 2. Mass spectrometer MS^1 chromatogram showing the overlaying of fentanyl peaks $[M + Na]^+$ detected in 24 dolphin blubber samples with fentanyl present

Peaks represent the intensity signal of the analyte when detected by the instrument.

not have access to non-blubber tissue matrices from the post-mortem dolphins sampled. Sampling live-stranded animals that are subsequently euthanized may add insights of exposure levels if plasma, liver, kidney, or brain tissues can be assessed.

Because meprobamate is a metabolite of carisoprodol, it is unclear whether the dolphin in our study had metabolized carisoprodol or had been directly exposed to it. Only three pharmaceuticals were tested and were selected based on results of an untargeted analysis. Each pharmaceutical assessed was present in at least one dolphin, but no dolphin had multiple pharmaceuticals detected. Testing additional pharmaceuticals could yield more CECs per dolphin. A statistical analysis was not possible due to the low number of blubber samples obtained and lower number of samples with pharmaceuticals detected. It was also not possible to control for different sampling years and unequal sampling efforts across study sites, which limited the scope of comparisons between locations.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Dara Orbach (dnorbach@gmail.com).

Materials availability

This study did not generate unique reagents.

Data and code availability

Data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

D.N.O., M.A.G., H.A., and C.W. conceived the idea. C.S., M.A.G., and D.N.O. collected the data. A.I.O. and M.A.G. prepared the samples. A.I.O., M.A.G., H.A., and J.E. analyzed the data. D.N.O. and A.I.O. wrote the manuscript with edits from M.A.G., J.E., C.S., C.W., and H.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- METHOD DETAILS
  - Study sites
  - Remote biopsy sampling
  - Standards preparation
  - Instrumental analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Data analysis and quantification
  - Limit of detection (LOD) and limit of quantification (LOQ)

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
N/A- wild animals	N/A- wild animals	N/A- wild animals

METHOD DETAILS

Study sites

The Redfish Bay (RB) system, Texas, consists of dolphins inhabiting the inshore waters of Port Aransas, Texas, and extends to Aransas Bay, Texas. The survey area is approximately 260 km^2 with an average water depth of 4.3 m.[46] RB has one point of outflow to the Gulf of Mexico (GoM) near the town of Port Aransas. The RB system is connected to Corpus Christi Bay in the southwest.[46] The 2022 population abundance estimate of dolphins inhabiting RB is 1,104 dolphins (unpublished data). Peak abundance occurs in winter months and many dolphins are year-round residents.[52] Corpus Christi Bay connects to Laguna Madre (LM) through the narrow enclosed Intercoastal Waterway that spans 443 km in length and 3-6 km in width, with an average water depth of 1 m.[46] The Upper Laguna Madre (ULM) is the northern part of the LM system that extends from Corpus Christi Bay in the north to the land cut in the south, spanning 80 km. ULM connects to Baffin Bay in the west and there is no outflow of ULM to the GoM. The survey area of ULM is approximately 46 km^2. Little research has been conducted on the dolphins inhabiting ULM.[46,53] At least 408 different dolphins have been photo-documented between 2019-2022 (unpublished data). The Mississippi Sound (MS) is located off the Mississippi coast, extending to Lake Borgne, Louisiana and Mobile Bay, Alabama, and bordered by the barrier islands of the Gulf Islands National Seashore to the south for a total area of approximately 4,792 km^2.[54,55] MS varies in width from 7.2-22.5 km and has an average water depth of 3 m.[54] The surveyed area is approximately 643 km^2. Dolphins inhabited MS exhibit seasonal spatial distributions, with peak densities during the summer.[55–57] The population abundance estimate is 1,265 dolphins in MS.[58]

Remote biopsy sampling

Research was conducted under NOAA NMFS permits 21938, 779-1633, and 14450, and Texas A&M University-Corpus Christi's Institutional Animal Care and Use Committee (IACUC) permit 2021-10-031. Blubber samples were obtained from distinct stocks of dolphins inhabiting Redfish Bay (RB) and Upper Laguna Madre (ULM), Texas, and Mississippi Sound (MS), Mississippi (Figure 1). Remote biopsy surveys were conducted in RB from 2012-2014 and 2022, in ULM during 2022, and in MS during 2013. Historic samples from 2012-2014 were used to explore the prevalence of pharmaceuticals over time. Research vessels included a 6.4 m Sea Fox with 200 HP motor, 7 m Boston Whaler Outrage with twin 150 HP motors, and 6 m rigid hull inflatable Inmar with 90 HP motor. Biopsy samples of dolphin blubber were collected using a crossbow (Barnett Panzer V, 68 kg draw weight) and custom-designed floating darts (Ceta-Dart) that rebound upon penetration of blubber.[59] A 7 × 25 mm or 10 × 25 mm stainless steel dart sampling tip held the tissue sample after penetration.[58] In between uses, tools including sampling tips were washed with antibacterial soap, rinsed, soaked in a 10% bleach solution for 10 minutes, rinsed again in deionized water, and autoclaved for 90 mins at 121°C (15 psi). Dolphin groups with a neonate or calf < 1 year were avoided per federal permit regulations. Initial GPS coordinates were recorded upon approaching dolphin groups. Samples were obtained from the flank of the dolphin, 3-4 inches below the dorsal fin and above the dolphin's midline. The sampling distance was typically 3-7 m from the target animal.[59]

Blubber samples were immediately retrieved from the darts and processed on ice using a cutting board covered with a sterile Teflon sheet. Sterilized scalpels and forceps were used to separate the blubber from skin. Samples were stored in vials sterilized by the National Institute of Standards and Technology in a liquid nitrogen vapor shipper until return to land and transferred to a -80°C freezer until processing. Metadata including environmental parameters, dolphin group composition, and biopsy data were recorded. Additional blubber samples were collected opportunistically from post-mortem dolphins (mostly fresh dead) that stranded in 2022-2023 in or near Redfish Bay and Upper Laguna Madre and were stored in -20°C freezers.

Standards preparation

All glassware was initially soaked in a 5% Tergazyme solution overnight, rinsed with tap water, washed with deionized (DI) water, soaked in 5% HCl for 12 hours, followed by further rinsing with DI water and Milli-Q ultrapure water. Glassware was subsequently oven-dried and combusted at 450°C for 12 hours. Immediately before use, glassware was rinsed with LC-MS Optima-grade acetonitrile (ACN). Procedure blanks ($n = 3$) underwent the same extraction processes but without the addition of the test blubber samples to ensure no contamination.

A combination of targeted and untargeted approaches were employed[60] to identify which pharmaceuticals to assess (Methods S1). Both non-isotopically labeled pharmaceutical standards (fentanyl, carisoprodol, meprobamate, Sigma-Aldrich) and isotopically labeled pharmaceutical standards (Fentanyl-$^{13}C_6$ with purity 99.5%, with 0.00% $^{13}C_0$ vs $^{13}C_6$, 3.84% $^{13}C_5$, 0.09% $^{13}C_4$ and 96.08% $^{13}C_6$; Carisopropdol-$^{13}C_3$ with purity 99.5%, with 0.00% of $^{13}C_0$ vs $^{13}C_3$, 2.58%, $^{13}C_2$ 97.43%; Meprobamate-$^{13}C_3$ with purity >98.7%, with 0.00%$^{13}C_0$ vs $^{13}C_3$, 2.89% $^{13}C_2$, 0.01% $^{13}C_1$ and 97.10%$^{13}C_3$) were used. The labeled standards were prepared in LCMS Optima-grade acetonitrile (ACN) at an initial concentration of 1 mg/mL (1,000 ppm). These standards were then combined to form a working solution with a final concentration of 1 ppm. The isotopically labeled pharmaceutical standards were used solely to validate the calibration curve and for spiking in the precision and recovery study. The isotopically labeled pharmaceutical standards were not spiked during sample analysis to prevent potential false positive detections from residual non-isotopically labeled standards. Successive serial dilutions were performed from the working solution to generate a calibration curve with concentrations ranging from 0.001 to 500 ng/mL. For the isotopically labeled standards, an additional dilution with a final concentration of 20 ng/mL was prepared for spiking test samples during the precision and recovery study (Methods S2).

The results of the precision and recovery study informed the decision to apply the same homogenization and pharmaceutical extraction method to all blubber samples, using a fixed mass of 150 mg blubber in ACN (Methods S2). Following extraction, all samples were reconstituted in 150 μL of ACN. Additionally, a blank sample of ACN was treated and analyzed multiple times during the sequence to ensure the accuracy of the result. Blubber homogenization and pharmaceutical extraction used combined techniques.[61,62]

Instrumental analysis

A Thermo Fisher Vanquish UHPLC system coupled with an ACQUITY UPLC BEH C18 reverse-phase column (130 Å, 1.7 μm, 2.1 mm × 150 mm) and an Orbitrap Fusion Tribrid mass spectrometer were used for the analysis. The mass spectrometer was operated with the Orbitrap as a high-resolution mass analyzer, the quadrupole as a mass filter, and the ion trap for fragmentation spectra analysis. Pharmaceutical concentrations were quantified by running analytes extracted from blubber samples alongside non-labeled external standards. To avoid carryover between samples or from standards to samples in the LC-Orbitrap MS, the column is placed in 100% ACN for 3 min at the end of each analysis followed by a 7 min re-equilibration between runs to remove any residual carryover. We ran analysis blanks (ACN) at the beginning of each batch, after the calibration curve (twice), and after every 10 samples to check for potential carryover. Additionally, we analyzed procedure blanks (n=3) as samples, which serve as a control and consist of vials subjected to the same steps of the analytical procedure, including sample preparation, extraction, and analysis, but without the addition of blubber. In all blanks (both analysis and procedure), none of the targeted pharmaceutical compounds were detected.

Chromatographic conditions

The mobile phases consisted of Milli-Q (MQ) ultra-pure water with 1% formic acid (A) and acetonitrile (ACN) with 1% formic acid (B). The chromatographic separation was performed at a flow rate of 0.200 mL/min with the total run lasting 31 min with a 7 min re-equilibration and the following gradient: 0–2 min hold at 5% B, ramp to 65% B for 18 min, ramp to 100% B for 1 min, and hold at 100% B for 3 min. Samples were injected at a volume of 5 μL.

Mass spectrometry conditions

UHPLC element was ionized using heated electrospray ionization (H-ESI) at 3,500 volts in positive ion mode. The ion transfer tube temperature was set to 300°C and the vaporization temperature to 225°C. Gas flow rates were set at 35 for sheath gas, 7 for auxiliary gas, and 0 for sweep gas. The Orbitrap operated at 60,000 FWHM resolution (at 200 m/z), with a mass range of 85–700 m/z and an RF lens setting of 40%.

For each Orbitrap acquisition cycle, we prioritized acquiring MS/MS fragmentation spectra of three pharmaceuticals using the ion trap, focusing on their specific retention times. Secondary priority was assigned to two additional MS/MS scans, performed using a data-dependent acquisition (DDA) approach with a quadrupole isolation window of 0.7 m/z. The first MS/MS scan employed collision-induced dissociation (CID) with an automatic gain control (AGC) target of 3.0×10^4 and a maximum injection time of 50 ms. The second MS/MS scan used higher-energy collisional dissociation (HCD), with collision energy optimized through real-time assisted optimization at 15, 30, and 45 eV, and an AGC target of 1.0×10^4. By incorporating reference standards to confirm retention times and ensuring high mass accuracy, we confidently assign our identifications to Level 1.[63]

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis and quantification

TraceFinder 5.1 (Thermo Scientific) was used to determine analyte peak areas and retention times for the $[M+H]^+$ and $[M+Na]^+$ adducts. Concentrations were calculated by applying the peak areas to the analytes' standard calibration curves. Percent recovery and precision (standard deviation) were determined for each analyte.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was established by performing 9 replicate injections, ensuring a signal-to-noise (S/N) ratio > 3, a mass error < 5 ppm, isotope patterns, and a retention time deviation within ±0.4 min of the corresponding standard. Multiple adducts ($[M+H]^+$ and $[M+Na]^+$) were evaluated, with the lowest LOD value selected.

For LOQ, a S/N ratio > 10 was required, along with MS/MS fragmentation spectra collected in the ion trap. LOQ determination also required a mass error < 5 ppm and retention time detection within ± 0.4 min of the pharmaceutical standard and a minimum of three matching fragments were required for confirmation.