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Detection of lithium in breast milk and in situ elemental analysis of the mammary gland

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Abstract: Breast feeding provides considerable benefits to the infant and mother. However, a lithium-based psychiatric medication may cause side effects in the child. Using laser induced breakdown spectroscopy (LIBS), trace lithium levels were observed in the breast milk of lactating rats administered with lithium treatment postpartum. Subsequently, the mammary glands of female rats were analyzed using LIBS, energy dispersive X-ray fluorescence spectroscopy, and inductively coupled plasma mass spectrometry. Key biological elements iron, magnesium, cobalt, calcium, phosphorus, sodium, iodine, potassium, sulfur, chlorine and zinc were observed. Lithium at 1.06 µg/g was measured in the mammary glands of treated subjects, but was below the limit of detection in controls. Lithium also increased iodine content in the glands. Lithium is present in the breast milk and mammary glands of lithium treated female subjects and this is the likely route of entry to breast-fed infants.

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References and links
22. X. Huang, “Does iron have a role in breast cancer?” Lancet Oncol. 9(8), 803–807 (2008).
1. Introduction

Breast milk is a natural and nutritious food source for infants and can protect them from disease while their own immune systems mature [1]. Breast milk contains complex proteins, lipids, carbohydrates and other biologically active components [2]. These components are capable of inhibiting inflammation as well as enhancing specific antibody production [3,4]. Because of the benefits, the World Health Organization and UNICEF recommend breast feeding infants at least for two years. However, the milk composition can change over a single feed, as well as over the lactation period, and depends on the health of the mother [2]. Many popular beliefs regarding the diets of lactating mothers have evolved from observations of side-effects produced in infants, and some scientific knowledge has been acquired in this regard [5]. An important issue has been the recognition that the fetus and newborn infant may be the unintended recipient of drugs administered to the pregnant or postpartum women. As a general rule, medication of the same class are expected to behave similarly in infants if they are unintentionally transferred through breast milk [6].

Breast milk contains important macronutrients calcium (Ca), magnesium (Mg), sodium (Na), phosphorus (P), and sulphur (S) and micronutrients cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se), and zinc (Zn). Breast milk also contains some potentially toxic elements silver (Ag), arsenic (As), boron (B), barium (Ba), cadmium (Cd), cesium (Cs), lithium (Li), lead (Pb), rubidium (Rb), antimony (Sb), strontium (Sr), uranium (U), and vanadium (V) [7–9]. These elements are also normally present in the mammary glands of the mother, which produces the milk. The mammary glands are capable of regulating the concentrations of essential elements such as Cu, Fe and Zn in milk to protect the newborn infant against deficiency and excess [10]. However, knowledge regarding toxic elements in breast milk, such as how they appear, interact, or are affected by maternal intake, is limited. Therefore, analyzing the elemental composition of breast milk and tissues under normal circumstances and with the mother on medication would provide clinically valuable knowledge.

Elemental analyses have reported macro and micro nutrients present in breast milk primarily using mass spectroscopy techniques. A wide variation has been reported in these nutrients across the globe [11]. Deficiencies in these nutrients have been correlated with the...
dietary intake of the mother. Elemental analysis has also been performed on breast tissue during cancer [12–14], focusing on calcification [15–17]. In addition to breast tissue calcification [15,16], previous research on elemental composition of breast tissue has focused on iodination [18] and efficiency or deficiency of zinc [19–21] and iron [22,23].

Lithium medication has been used extensively to treat bipolar disorder [24]. The global prevalence of bipolar disorder is 2.4%. Due in part to the low atomic weight of lithium, its biodistribution has been relatively poorly characterized in tissues and organs. Relatively few studies have been conducted examining the effect that lithium has on breast cells [25,26] and the lactation processes [9,27]. Lithium medication is also associated with thyroid [28] and kidney dysfunction [29]. In spite of these previous findings, no study has reported lithium distribution in the body, except in the brain and in serum, with capillary ion analysis [30] and neutron capture reaction [31], respectively.

Laser induced breakdown spectroscopy (LIBS) and energy dispersive X-ray fluorescence (XRF) are both elemental analysis methods sensitive to low and high atomic weight elements, respectively [32,33]. The LIBS technique employs a high intensity laser pulse to ablate a small volume (µm³) of sample [34], whereas XRF collects secondary X-rays from the sample when the primary X-ray source irradiates it. The LIBS and XRF instrumentation are relatively compact, inexpensive, and do not require destroying the sample. This allows relatively straightforward in situ biochemical analysis of samples. Also, LIBS and XRF can be used in conjunction with microscopy to examine microstructure and biochemistry in the same sample. LIBS and XRF are complementary methods as together, they are sensitive to elements across much of the periodic Table [35]. This combined approach has provided fast and multi-element analysis of plant nutrition [35] and our group has recently explored dental applications [36]. Also, LIBS can analyze solid, liquid, and gaseous samples while XRF is primarily for solid samples. In this study, we exploit the complementary abilities of LIBS and XRF to analyze a wide range of elements in liquid breast milk samples and solid mammary gland samples. This will help to understand the entry route of lithium into breast milk.

The primary objective of the present study is to detect trace lithium in the breast milk of lactating rat subjects on lithium treatment. Subsequently, the impact of lithium on the mammary glands is investigated with multimodal in situ elemental analysis. Rats were chosen since the mammary glands of humans and rats are similar in structure and function [37,38]. This objective is important as lithium in breast milk, which is produced by the mammary glands, is the likely conduit of transmission for lithium into breast-fed babies. This study will also help establish the rat as a model for lithium biodistribution studies. The secondary objective of this study is to present LIBS and XRF as complementary, inexpensive, and easy to implement methodologies for trace elemental analysis of breast milk and tissues.

2. Material and methods

2.1 Animal subjects

Female Sprague Dawley (SD) lactating rats at two weeks postpartum (N = 2, 400 – 450 g) and virgin female SD rats at 10 weeks age (N = 10, 275 – 300 g) were employed for the study. The lactating rats each nursed 12 pups, which otherwise did not take part in this study. Subjects were provided by the AAALAC accredited Laboratory Animal Unit of the University of Hong Kong. This study was approved by the animal research ethics committees of the City University of Hong Kong, the University of Hong Kong, and the Department of Health of the Hong Kong Special Administrative Region. Each lactating subject was housed individually, with her pups, while female subjects were housed five per cage. Housing was at a constant temperature of 25 °C and humidity of 60 to 70% in the Laboratory Animal Research Unit of the City University of Hong Kong. All subjects were housed in 12/12 hour light/dark cycles and had access to regular chow food and drinking water.
2.2 Lithium medication preparation and administration

Lithium carbonate (Li₂CO₃) was purchased from Sigma Aldrich (USA). For the lithium treatment group (N = 6, one lactating and five female subjects), 1000 mg of Li₂CO₃ per kilogram of body weight was the daily dose. Before administering Li₂CO₃, subjects were weighed daily. Following the weight measurements (see Table 1), subject specific 1 ml solutions of Li₂CO₃ were prepared in individual 10 ml tubes. The Li₂CO₃ salt was dissolved into distilled water with the help of magnetic stirring for 30 minutes at room temperature. Later, the solutions were fed to the respective subjects using gavage. This daily regimen of lithium treatment was continued for two days for lactating subjects (postpartum days 18 (8.2 mg/411g) and 19 (8.1 mg/405g)) and seven days for female subjects. Control subjects (N = 6) were similarly gavaged with water only.

<table>
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<th>Days</th>
<th>Rat 1 (g)</th>
<th>Dose (mg/ml)</th>
<th>Rat 2 (g)</th>
<th>Dose (mg/ml)</th>
<th>Rat 3 (g)</th>
<th>Dose (mg/ml)</th>
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</table>

2.3 Sample preparation

On the 20th day postpartum, lactating rats were anesthetized using isoflurane (3%), sterile alcohol prep pads were used to clean the breast area of the subjects, and sterile eye lubricant were used to prevent dryness of the eyes. Breast milk was extracted by manually squeezing the breasts from both control and lithium treated lactating subjects (Fig. 1). Approximately 60 μL of milk were collected in total from each subject and stored in multiple 20 μL capillary tubes. After milk collection, subjects, along with their pups, were euthanized by 1 ml/kg body weight of 20% Dorminal administered via intraperitoneal injection. Later, the milk was dried for 48 hours on weighing paper in standard atmosphere in preparation for LIBS measurements. The paper was pre-checked with LIBS to be free of trace lithium.

On the 8th day, after seven days of gavage, female lithium treated and control subjects were euthanized by Dorminal overdose. Note that virgin female subjects were employed in this step, rather than lactating subjects, for ethical reasons to considerably reduce the total number of animals used in the study. The mammary glands (breast tissues) were extracted from the subjects using surgical tools. This tissue was located on the thorax in between the forepaws of female rats (Fig. 1). The excised mammary glands were immersed in saline to
remove blood. The tissues were then placed in 50 ml test tubes and immersed in liquid nitrogen for several seconds. After liquid nitrogen, the samples were transferred onto glass slides with pre marked positions to facilitate measurement. The samples were stored in a −80 °C freezer for 24 hours followed by an additional five days of freeze drying. The freeze drying was performed using Labconco’s FreeZone Plus at −70 °C temperature and 0.42 mbar pressure to ensure proper dryness of the sample before acquiring XRF and LIBS measurements.

After acquiring LIBS measurements, all mammary glands of female, lithium treated subjects were combined to make a single sample of 50 mg for quantitative measurement of lithium concentration using inductively coupled plasma mass spectrometry (ICP-MS) with microwave assisted acid digestion. The glands of control subjects were similarly combined and lithium measured.

2.4 Experimental setup

The energy dispersive XRF apparatus (Edax’s Eagle III), illustrated in Fig. 2a, was used first to analyze the mammary glands with the following parameters: voltage 40 kV, current 600 mA, amplification time 6 µs, and acquisition time 60 seconds with no filter. A low-power high-voltage generator was used to operate the X-ray tube. The X-ray source was a molybdenum tube operated in vacuum condition. XRF measurements were acquired from the center of each left and right mammary gland. After XRF measurements, the gland samples underwent LIBS.

Figure 2b shows the setup of LIBS, which was used to analyze breast milk and mammary glands. The 1064 nm pulsed laser (CFR200, Quantel) emitted 8 ns, 200 mJ pulses focused to a 10 µm spot on the sample. The optical emission from the sample was collected by a six channel fiber (2000 µm diameter) bundle, positioned 35 mm from the focus and at 45° from the laser beam. The fibers relayed light to six spectrometers spanning 200 – 900 nm with 0.1 nm resolution (MX2500 +, Ocean Optics). The spectrometer was triggered to acquire 2 µs after laser firing and with 1 ms acquisition. Measurements for LIBS were obtained in standard air atmosphere by placing the center of each dried milk sample (along with paper) and mammary gland at the laser focus and firing three shots. Emission spectra for the shots were recorded separately. A PC synchronized the setup and processed spectral data acquired by the spectrometers, producing a graphical presentation of spectral intensity against the corresponding wavelength.

For quantification of lithium in mammary glands, ICP-MS (Perkin Elmer DRC II) was employed as per the protocol of AOAC INTERNATIONAL. The combined samples were digested using HNO₃ and H₂O₂ and ICP-MS was performed as per the protocol [39]. Gland samples from different subjects had to be combined for this quantitative measurement as the
expected Li concentration was low (ppm level) and each gland weighed approximately 10 mg.

2.5 Data analysis

The raw XRF spectra were imported to OriginLab software for baseline correction. After baseline correction, peaks were identified from the Harvard X-ray emission database. The XRF spectra for left and right mammary glands were averaged to obtain an average spectrum for each subject along with standard deviation. Each averaged spectrum was then normalized by the respective quantum scattering lines obtained at 1637 eV. For LIBS measurements, the spectrometer was calibrated using argon gas and mercury lamp (PhyWe) spectra. The argon and mercury spectra were matched with spectral lines available in the Atomic Spectra Database of the National Institute of Standards and Technology. Calibrated spectra were also matched with the database for elemental analysis. The raw data from the LIBS spectrometer was imported into OriginLab software for baseline correction. Next, the spectra from the three laser pulses fired at each milk sample were averaged to obtain the spectrum for each lactating subject along with standard deviations. Similarly, the spectra from the six laser pulses (three for left and three for right gland) fired at each female subject’s mammary glands were also averaged to obtain the spectrum for each subject along with standard deviations. The intensity of each mammary gland LIBS spectrum was normalized to the 656.2 nm hydrogen line since the line has high intensity and hydrogen is a structural element of the gland not likely to be significantly affected by lithium treatment. Statistical analysis of the spectra obtained from the mammary glands was performed using one way ANOVA across the elements and subject groups. A post hoc analysis using two tail t-test of equal variance was performed. A p-value threshold of 0.05 was considered statistically significant. Correlation coefficients (cc) were calculated across the groups for the intensities of common elemental emissions between LIBS and XRF. This quantifies correlations between LIBS and XRF measurements. For quantification of lithium using ICP-MS, a calibration curve was plotted from different concentrations of lithium. Then the mammary gland’s Li concentration was obtained from the calibration curve.

3. Results

Figure 3(a) shows the unnormalized LIBS spectra obtained from milk samples of a control (black) and a lithium treated lactating subject (red) expanded about 670 nm. A Li line is observed in treated subjects only at 670.7 nm.

Figure 3(a) shows the unnormalized LIBS spectra obtained from milk samples of a control (black) and a lithium treated lactating subject (red). The control spectrum does not show any emission line while the treated spectrum shows a prominent line at 670.7 nm, which belongs to lithium. This shows the presence of Li in the breast milk. The unnormalized intensity for lithium in breast milk is 271 ± 67 from the treated subject. Lithium was found in breast milk of humans using ion-selective electrode detection [9], however no comprehensive studies have been reported on the impact of lithium on the lactation processes [9,27] and on the mammary glands.
Fig. 4. (a) Averaged LIBS spectrum from the six shots fired at the left and right mammary glands of a control subject. (b) Magnesium (Mg) emission lines were observed at 279.6 and 280.3 nm. (c) Cobalt (Co) emission was observed at 388.3 nm. (d) Calcium (Ca) was observed at 393.4, 396.9 and 422.7 nm. (e) Sodium (Na) lines were observed at 589.0 and 589.5 nm. (f) Iodine (I) line was observed at 746.9 nm. (g) Potassium (K) lines were observed at 766.4 and 769.9 nm. Error bars indicate standard deviation of intensities across shots at the respective emission lines.

Figure 4 shows the LIBS spectrum obtained from mammary glands (left and right) of a control subject. Figure 4(a) shows the whole spectrum from 200 to 800 nm. There were several prominent emission lines showing the spectral signature of different elements. The sub-panels, Figs. 4(b-g) show the spectrum expanded about different wavelength ranges. Magnesium emission lines were observed at 279.6 and 280.3 nm. Cobalt emission was observed at 388.3 nm. Calcium lines were observed at 393.4, 396.9 and 422.7 nm. Sodium lines were observed at 589.0 and 589.5 nm. An iodine line was observed at 746.9 nm. Potassium lines were observed at 766.4 and 769.9 nm. These are important elements for proper functioning of the mammary gland [40]. A prominent hydrogen line was observed at 656.2 nm. Amongst the key trace elements, Na lines had the highest intensity, followed by K, Co, Mg, I, and Ca. The un-normalized intensity (mean ± standard deviation) of the six shots from one control subject for Mg, Co, Ca, Na, I, and K were 977.4 ± 355.5, 1516 ± 458, 591.4 ± 175.6, 8192 ± 1565, 748.1 ± 196.3, and 5412 ± 1208 a.u., respectively.

Fig. 5. The XRF spectrum obtained from a control subject averaged across the left and right mammary glands. Peaks corresponding to phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca), iodine (I), iron (Fe), cobalt (Co) and zinc (Zn) were clearly visible. All emissions above were from the K shell, except for iodine, which came from the L shell.

Figure 5 shows the XRF intensity spectrum obtained from the mammary glands (averaged across left and right glands) of a control subject. The un-normalized peak intensities obtained from XRF spectra show clear peaks of phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca), iodine (I), iron (Fe), cobalt (Co) and zinc (Zn). The peak intensities along with standard deviation for P, S, Cl, K, Ca, I, Fe, Co and Zn were 224.2 ± 82.7, 716.8 ± 72.8, 348.9 ± 49.4, 348.3 ± 42.8, 192.3 ± 33.1, 60.6 ± 17.7, 280.9 ± 4.5, 55.5 ± 0.5 and 91.1 ± 28 a.u., respectively. The highest peak intensity was from S, an active biological element in animal tissues [41], whereas the lowest peak was from cobalt.
Figure 6 shows the normalized and group averaged LIBS spectra of mammary glands from control and lithium treated subjects. The spectra have been normalized by their respective hydrogen emission lines at 656.2 nm. Figure 6(b-h) shows the spectra expanded about Li, Mg, Co, Ca, Na, I and K lines, from both control (in black) and lithium treated groups (in red). No lithium emission was observed in the averaged control spectrum expanded in Fig. 6(b). With the intake of lithium, a significant lithium emission line (t_{1,8} = 3.27, p = 0.01) was observed at 670.7 nm. Also with the intake of lithium, iodine (t_{1,8} = 2.4, p = 0.04) intensity was increased (Fig. 5(i)). The intensities of Mg, Ca, Na and K and Co elements were also affected, but not significantly.

Figure 7 shows the group averaged XRF spectra obtained from the mammary glands of control subjects (N = 5) and 7 day lithium treated subjects (N = 5). Each subject’s spectrum was normalized first by the respective quantum scattering lines at about 1637 eV and then averaged for both mammary glands. Figure 7 (b) shows the spectra expanded about P, S, Cl, K, Ca, I, Fe, Co and Zn. The spectra were normalized by the quantum scattering lines obtained at around 1637 eV. From XRF measurements, Ca/P ratio for control group was found to be 0.90 ± 0.80, which was increased to 1.27 ± 0.60 in lithium treated group.
Fig. 8. (a) Bar plots showing the mean and standard error of LIBS emission intensities for Mg, Co, Ca, Na, Li, I and K from the mammary glands of control (N = 5) and 7 day lithium treated subjects (N = 5). (b) Bar plots of XRF emission intensities for P, S, Cl, K, Ca, I, Fe, Co and Zn from control and lithium treated subjects. Statistical analysis between groups was performed with one way ANOVA. A post hoc analysis using two tail t-test of equal variance was performed with p-value threshold of 0.05 considered statistically significant. * indicates p<0.05. Cross correlation analysis was also performed to compute the correlation coefficients (cc) between the common elemental emissions from LIBS and XRF.

Figure 8(a) shows the bar plots with mean and standard error of LIBS emission intensities of Mg, Co, Ca, Na, Li, I and K from the mammary glands of control and 7 days lithium treated subjects. LIBS intensities have been normalized by the 656.2 nm hydrogen line intensity. From LIBS measurements, the changes in intensities of lithium and iodine were found to be statistically significant (p<0.05) with the intake of lithium. Iodine in mammary gland increases with accumulation of lithium. The accumulation of lithium in the mammary glands was quantified using ICP-MS, which was observed to be 1.06 μg/g (1.06 ppm) for treated subjects and below limit of detection for controls. Figure 8(b) shows the bar plot with mean and standard deviation for XRF emission intensities of P, S, Cl, K, Ca, I, Fe, Co and Zn from the mammary glands of control and 7 days lithium treated subjects. However, none of the elements differed statistically significantly between the two groups. The cross correlation analysis was also performed to compute the correlation coefficients (cc) within common elements observed in LIBS and XRF. The corresponding correlation coefficients for Ca, I, and K were cc = 0.76, 0.50, and 0.36, respectively. Cobalt showed little correlation between the two methods with cc = −0.01.

4. Discussion

The present study detected trace lithium in the breast milk of lactating rats administered lithium carbonate. Subsequent in situ elemental analysis of the mammary glands of control and lithium treated virgin female rats, primarily with LIBS and XRF, also detected lithium. The accumulation of lithium in the mammary glands was validated by acid digestion ICP-MS. The concentration in treated subjects was 1.06 ppm and below limit of detection in controls. The LIBS measurements showed statistically significant differences among key elements lithium and iodine in the mammary glands. Iodine was increased by lithium. The LIBS and XRF measurements were validated against each other through correlation analysis. The correlation coefficients indicated a positive trend for most elements commonly measured by LIBS and XRF (Ca, I, and K), suggesting that the methods are in agreement.
Figure 9. (a) Illustration of thyroid and mammary gland and the blood vessels connecting both organs. (b) Model showing hypothesized mechanism for accumulation of iodine in the mammary glands and concurrent reduction of iodine in the thyroid [28]. Excess iodine in the blood due to the presence of lithium causes extra iodine to enter the mammary glands via the sodium iodide symporter (NIS).

Figure 9 shows the hypothesized mechanism for accumulation of iodine in blood serum [42] through the sodium iodide symporter (NIS) caused by lithium treatment, and its possible transport to the mammary glands through the same NIS expression. Significant increases of iodine in mammary glands, as observed in this study, is likely related to the uptake and utilization of dietary iodide mediated by NIS expression [43,44]. NIS is expressed in the mammary, salivary, and thyroid glands [45]. Coincidentally, lithium also affects the salivary glands and thyroid [46] and breast cancer has been observed to be related to thyroid disorders [18]. During lithium treatment, lithium ions may enter the thyroid through the sodium/potassium ion channel as they all exist as positive ions. Accumulation of lithium ions in the thyroid may decrease the iodide influx from the blood vessel to the cells of the thyroid [47]. Conversely, iodine level in the salivary glands was found to be enhanced, similar to the mammary gland findings of this study, through NIS expression [46]. We hypothesize that a significant increase of iodine in the mammary glands occurs due to an abnormal production of thyroid hormones caused by lithium (Fig. 8). Support for this hypothesis comes from observations that lithium decreasing thyroid hormones concurrently increases iodine in serum [28]. The excess iodine is transported to mammary tissue through NIS expression, resulting in the significant increase of iodine (p<0.05) with lithium observed in this study, analogous to in the salivary gland [46].

There are several technical limitations in this study. (1) The sampling volumes of LIBS and XRF are different. LIBS sampling depends on the energy and number of laser shots while XRF sampling depends on photon energy. In general, LIBS samples more superficial parts of the sample while XRF samples deeper parts. Also, the sample preparation method affects the sampling volume, since the sample is soft tissue, and the effect may be different for LIBS and XRF. However in this study, the spatial scales range from 100 µm to millimeters and mammary gland tissue is relatively homogeneous in controls on this scale [48]. This is also likely the case in lithium treated tissues. (2) Quantitation of low concentrations by LIBS, such as the lithium concentration, is challenging due in part to plasma inhomogeneity. To partially overcome this, we employed acid digestion ICP-MS on the mammary gland samples. This approach cannot be directly applied on the small quantity of rat breast milk samples.

Lithium use in pre-partum has been associated with a number of negative effects in the newborn including depressed neurological status, hypotonia, respiratory distress syndrome, cyanosis, lethargy, and weak suck reflexes [49]. These problems were typically resolved by reducing the pre-partum dose [50]. Current practice guidelines in post-partum discourage use of lithium during breast-feeding due to transient abnormalities of thyroid-stimulating hormone, blood urea nitrogen, and creatinine observed in infants [9]. Though the data set is
limited to ten subjects and no significant adverse clinical or behavioral effects in the infants were noted. In another study, three subjects were administered with lithium during postpartum and two infants experienced early feeding problems which were overcome with breastfeeding education and support [27]. The most recent publication classifies lithium as a drug that should be given to nursing mothers “with caution” [49], although there is little evidence beyond a handful of case reports [9,27]. Note that such a recommendation may deny the infant, and the mother, the many benefits of breast feeding [51]. LIBS, which is highly sensitive to the low atomic weight lithium element, will be very useful for expanding our understanding of the biodistribution of lithium. This will enable the development of better medication plans for breast-feeding Bipolar patients such that she receives proper treatment and both infant and mother can enjoy the benefits of breast feeding.

5. Conclusion

We applied LIBS and XRF to detect lithium in the breast milk of lactating rats and to perform elemental analysis of mammary glands from female rats administered lithium medication. From LIBS measurements, a lithium emission line was observed at 670.7 nm in the milk and glands of lithium treated subjects only. The lithium concentration in the mammary glands was 1.06 ppm. Iodine in glands was found to be significantly increased by lithium. There was positive correlation between the measurements of elements commonly observed by LIBS and XRF, suggesting the two methods are in agreement. Trace lithium in the mammary glands affects their overall elemental composition and this may be related to lithium’s effects on other organs such as the thyroid. This study has helped to validate the rat as an animal model for lithium biodistribution studies.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.