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Identification of multiple cardiotonic steroids in faecal material of untreated humans and rat strains

Zelie F. Masso [c,1], Hannah Bint Ebrahim Mullah [a,1], Anza Thiba [a], Sarhana Dinat [a], Ekene E. Nweke [a], Gavin R. Norton [b,2], Angela J. Woodiwiss [b], A. Duncan Cromarty [c], Geoffrey P. Candy [a,*]

[a] Department of Surgery, Faculty of Health Sciences, University of the Witwatersrand, Parktown, Johannesburg 2193, South Africa
[b] School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Parktown, Johannesburg 2193, South Africa
[c] Department of Pharmacology, Basic Health Sciences, University of Pretoria, Pretoria, South Africa

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ABSTRACT

Endogenous cardiotonic steroid (CTS) concentrations are raised in cardiovascular diseases. CTSs undergo gastro-hepatobiliary recirculation, with the gut being an important route of elimination, yet the presence of CTSs in faecal material is seldom reported. This study investigated methods to extract and identify the presence of CTSs in faecal material of rats and humans without prior treatment.

Methods: Freeze-dried faecal material from different untreated rat strains was extracted using various solvents, with separation and identification of CTSs using HPLC/MS. Preliminary results were obtained from human faecal material.

Results: Multiple CTSs were identified in faecal material, with marinobufagenin (MBG) predominant. Telocinobufagin was only detected in certain rat strains, whereas the extraction methods used did not recover ouabain. MBG and digoxin were elevated in Dahl salt sensitive rats fed supplementary salt. Bufalin was present in most spontaneously hypertensive rats (SHRs) but was not detectable in Wistar Kyoto rats (WKY). Conversely, digitoxin was detected in most WKYs but only few SHRs. Levels of digitoxin and bufalin remained relatively constant over 24 days in untreated rats. Solvent selection was critical in determining the CTSs extracted from human faecal material.

Conclusions: Multiple CTSs were detected in faecal material of untreated rats and humans. Steroids varied between rat strains and aligned with phenotype. Extraction requires further solvent optimisation and the use of tandem MS/MS is essential to reliably detect the profile of CTSs present. Analysis of CTSs present in readily available faecal material will enable studies to determine relationships between CTSs, the microbiome and disease progression.

1. Introduction

Cardiotonic steroids bind to a receptor site on the α-subunits of membrane sodium/potassium adenosine triphosphatase (Na/K-ATPase) ion transporters and regulate intracellular sodium and potassium ion concentrations, and whole-body sodium concentrations. The electrochemical gradient established across the cell membrane drives a host of cellular functions [1–3]. Independent of the ion transport function,

Abbreviations: AREC, Animal Research Ethics Committee; Na/K-ATPase, sodium–potassium triphosphatase; $CHCl_3$, chloroform; CTS, cardiotonic steroid; DSS, Dahl salt sensitive rat; ESI, electrospray ionisation; HPLC, high performance liquid chromatography; HDCA, hyodeoxycholic acid; IPA, isopropanol; LCA, lithocholic acid; MeOH, methanol; MTBE, methyl *tert*-butyl ether (MTBE), MRM, multiple reaction monitoring; MS, mass spectrometry; OLC, ouabain-like compounds; RP-SPE, reverse phase – solid-phase extraction; SHR, spontaneously hypertensive rat; SD, Sprague Dawley rat; m/v, mass/volume; v/v, volume/volume ratio; WKY, Wistar-Kyoto rat; WRAF, University of the Witwatersrand Research Animal Facility.

* Corresponding author.
E-mail address: geoffrey.candy@wits.ac.za (G.P. Candy).

[1] Zeelie F Masso and Hannah Bint Ebrahim Mullah contributed equally to this research.
[2] Deceased.

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cardiotonic steroid binding triggers signalling cascades that mediate cell-dependent processes [3,4].

Cardiotonic steroids (Fig. 1) are found in plants, insects, mammals and toxic amphibians. Immunoreactive ouabain-like compounds (OLC) produced endogenously were isolated and characterised from human plasma [5]. They may be elevated in cardiovascular and related diseases [3,6,7], volume-expanded, and salt-sensitive hypertension [8], and are associated with the manifestations of such diseases [7]. Concentrations of ouabain and marinobufagenin are increased in the plasma of humans with hypertension [9–13], myocardial infarction, and heart failure [12,14–16]. Ouabain has been associated with increased cardiac left ventricular mass [10] and remodeling [17], as well as aldosteronism [13]. Elevations in marinobufagenin [12] and plasma telocinobufagin [18] were reported in patients with renal failure, and telocinobufagin stimulated renal fibrosis through Na/K-ATPase-mediated signalling pathways [19]. Digoxin-like immunoreactivity, co-eluting with authentic endogenous ouabain and marinobufagenin, was increased in preeclampsia [20,21].

Pharmacokinetic studies of therapeutically important digoxin and related cardiac glycosides have provided valuable insight into the tissue distribution, excretion, and metabolism of cardiotonic steroids [22–24]. Orally administered digoxin was poorly absorbed, with up to 15 % recovered unaltered in the faeces [25]. The intestinal P-glycoprotein was important for regulating the absorption of orally administered digoxin from the intestine, as well as the direct efflux of intravenously administered digoxin into the intestine and has a significant role in regulating plasma digoxin concentrations [24,26]. Furthermore, although up to 50–85 % of orally administered digoxin can be recovered unchanged or as metabolites in the urine [22,24], bile is an important route of elimination of cardenolides, including digoxin, digitoxin, lanatoside C and ouabain [27].

The pharmacokinetics of bufadienolides are less well studied. After oral administration of traditional Chinese medicines, bufadienolides and metabolites have been identified in hepatic portal vein blood [28,29], plasma [29–33] and were widely distributed in mouse tissues, including the intestines, kidney, liver, and lungs [24]. A reported second time-separated peak in bufadienolide plasma concentrations is consistent with intestinal uptake and enterohepatic recirculation [30]. As with the cardenolides, bufadienolides were detected in rat bile [34–36], and bufalin immunoreactivity was demonstrated in human bile [37]. These data suggest the pharmacokinetics of bufadienolides are analogous to cardenolides, with bile being an important route of elimination.

The presence of unaltered orally administered cardiotonic steroids in the intestine would be expected following either poor absorption, entero-hepatobiliary circulation, with possible P-glycoprotein transport, and biliary excretion. Surprisingly, given the many pharmacokinetic studies of cardiotonic steroids, their presence in faecal material [4,6], and pre-administration faecal concentrations are seldom reported [30–32,36]. Such analyses would determine which cardiotonic steroids were present in faecal matter prior to administration of the cardiotonic steroid.

The present study attempted to address this by extracting and determining the presence of cardiotonic steroids in untreated spontaneously hypertensive (SHR) and control Wistar Kyoto (WKY) rats and provides preliminary data from human faecal material. The methodological and analytical limitations encountered when extracting and analysing unconjugated cardiotonic steroids from faecal material have been discussed in disease settings, with an emphasis on rat models of hypertension. Such analysis would permit studies to determine associations between these steroid profiles and a disease phenotype, which may provide insight into disease development and progression.

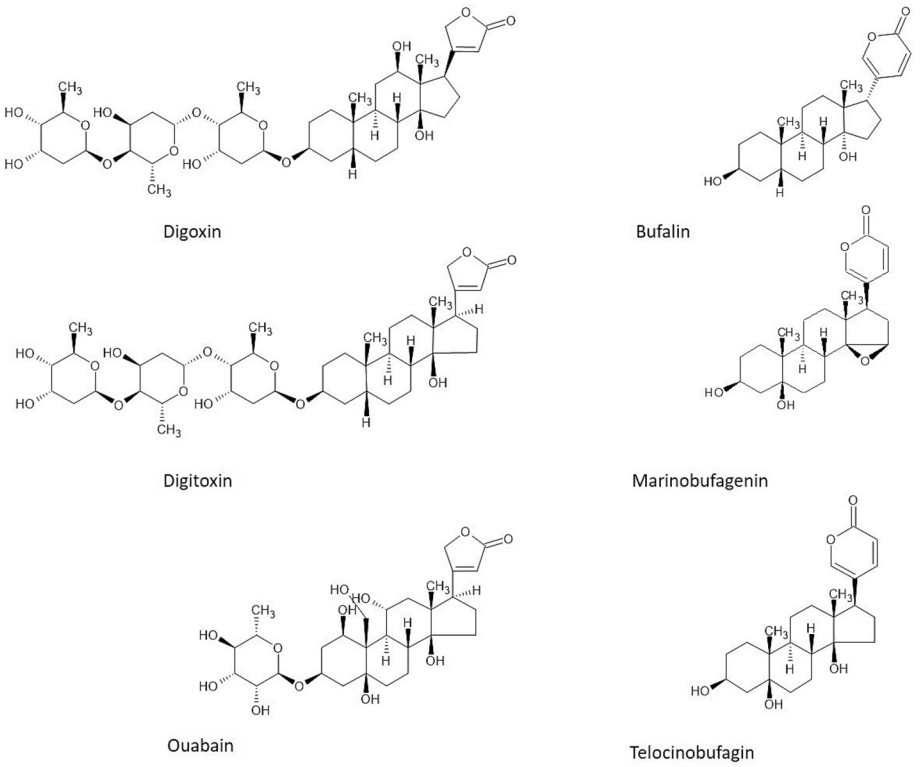

Fig. 1. Representative cardiotonic steroids mentioned in the script, showing cardenolides (left) and bufadienolides (right). Structures obtained from chemspider.com. Structure IDs as follows: Digoxin – 2006532; Digitoxin – 389987; Ouabain – 388599; Bufalin – 24534043; Marinobufagenin – 10142870; Telocinobufagin – 228185.

2. Methods

2.1. Ethical clearance

Briefly, three experiments were undertaken.

1) Ethical clearance was obtained from the Animal Research Ethics Committee (AREC) of the University of the Witwatersrand (AREC Approval 2018/09/42C with a Modification and Extension dated 29/05/2019) for experiments using SHR and WKY rats to determine changes in cardiotonic steroids over time and relationships of identified cardiotonic steroids with tail cuff systolic blood pressures.
2) To optimise the extraction of cardiotonic steroids from faecal matter used Wistar, Sprague Dawley (SD) rats, Dahl salt-sensitive (DSS) and spontaneously hypertensive rats (SHRs) being terminated in unrelated experiments measuring cardiac parameters. Permission to harvest the faecal samples from the lower gastrointestinal tract immediately upon termination of these rats, was obtained from the Animal Research Ethics Committee (AREC) AREC of the University of the Witwatersrand (Permission waiver letter dated 8 May 2017).
3) Preliminary data to compare solvents for extracting cardiotonic steroids in human faecal material, permission to collect faecal samples from 8 female control apparently healthy volunteers of African ancestry (aged 18–60 years) was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (M170582).
4) The development and optimisation of the HPLC/MS method to measure cardiotonic steroids in faecal matter (Points 2 and 3 above), permission was obtained from University of Pretoria (Ethics Reference No: 292–2017; 12 September 2019) as these results were included in the Master of Science dissertation (ZFM) undertaken in the Department of Pharmacology of the University of Pretoria, Pretoria, South Africa [38].

2.2. Animal studies

All procedures involving rats were conducted at the University of the Witwatersrand Research Animal Facility (WRAF) Unit of the University of the Witwatersrand, Faculty of Health Sciences, Parktown, Johannesburg, South Africa. WKY rats were imported from Charles River Laboratories GmbH (Sulzfeld, Germany) and the first and second offspring litters of WKY rats, bred in the WRAF Unit, were used. All SHR, Wistar, SD and DSS rats were bred in-house. In unrelated experiments measuring cardiac parameters, rats either had no intervention (Ctrl), 5 % (m/v) sodium chloride added to their drinking water (DSS-Salt), and the exercised SHRs (SHR Exercise; see Fig. 5) were given unrestricted access to a treadmill with a 1 m circumference in their cages at the WRAF Unit.

2.3. Laboratory procedures

2.3.1. Chemical reagents

Analytical standards bufalin, digitoxigenin, digitoxin, digoxin, ouabain, proscillaridin A and bile acids (Sigma Aldrich, St Louis, USA); marinobufagin, periplogenin, and telocinobufagin (Leapchem, Zhejiang, China) were used. Isotope-labelled digoxin-d3 standard was purchased from Santa Cruz Biotechnology (Texas, USA). Mass spectrometry (MS) reagents (purity > 99.9 %) included isopropanol (Fluka Analytical, Switzerland), acetonitrile, ethanol, methanol and other reagents and solvents (analytical reagent grade ≥ 98 %) (Merck, Darmstadt, Germany or Sigma Aldrich, St Louis, USA). Double-deionised pyrogen-free water of >18 MΩ produced in-house using an ELGA Genetics water purification system (High Wycombe, United Kingdom) was used.

2.3.2. Procedures involving animals

Three-month-old male (n = 3) and female (n = 3) WKY (total n = 6) and SHR rats (total n = 6) were housed individually with unlimited access to standard rat chow and water on a 12-hour light cycle with the temperature maintained at a constant 30 °C. The researchers checked the rats daily and each week they were weighed and checked by a qualified WRAF veterinarian or nurse.

The rats were initially habituated to handling and blood pressure recordings using a tail-cuff blood pressure monitor (NIPB250 Tail Cuff System; BIOPAC Inc., CA, USA). Blood pressures (SBP) were recorded in untreated animals twice per week over a 24 day period. Each evening when the rats were active, each rat was transferred into a separate metabolic cage (Labotec, Johannesburg, South Africa) and returned to standard housing each morning. Overnight faecal material separated from urine was collected each morning in sterile capped Falcon tubes and frozen at −80 °C until the bile acids and steroids could be extracted. The metabolic cages were cleaned thoroughly each day.

2.3.3. Extraction and analysis of rat faecal cardiotonic steroids

Faecal samples (1 g) were freeze-dried and extracted with methyl-tert-butyl ether [39,40] (MTBE; 10 ml) in the Department of Surgery laboratories. The faecal material was macerated with a spatula, homogenised in an ultrasonicated bath for two minutes, and placed on a rotatory shaker for an hour. After centrifugation, supernatants were collected into clean glass containers and evaporated to dryness under a nitrogen stream. Extracts were reconstituted in MTBE (5 ml) and stored at −80 °C until use.

Cardiotonic steroids extracted from these faecal extracts were analysed at the Shimadzu (South Africa) laboratory at the University of Johannesburg, Johannesburg. A Shimadzu Nexera X3 Binary UHPLC system with two LC-40D XS pumps was used to achieve separation on a 2.7 μm 4.6 x 100 mm Shimpak Velox biphenyl column. The flow rate was 0.3 ml/min with an oven temperature of 40 °C. The gradient is described in Supplementary Table S1. The chromatographic column was coupled to a Shimadzu LCMS-9030 Quadrupole Time of Flight (Q-TOF) detector (Shimadzu, Kyoto, Japan) with settings summarised in Supplementary Table S1. Bile acids were determined using the same HPLC column and parameters of the cardiotonic steroids with the acquisition polarity in negative mode, not positive mode (Supplementary Table S2).

Cardiotonic steroids are synthesised via bile acid intermediates [41,42] after cholesterol side chain cleavage [43] and bile acid turnover is low and tightly controlled [44]. To correct for dilution effects, the ratio of peak areas of the two positively identified cardiotonic steroids identified, digitoxin and bufalin, were determined relative to the areas of abundant bile acids detected: digitoxin or bufalin/(lithocholic acid + hyodeoxycholic acid + cardiotonic steroids). To determine variation over time, the ratios of bufalin (Fig. 2A) and digitoxin (Fig. 2B) were measured over the 24 day duration of the experiment. The relationship between ratios and blood pressure of the SHR and WKY rats were shown as scatterplots (Fig. 3).

2.3.4. Extraction optimisation of cardiotonic steroids from faecal material

In experiments to optimise the extraction of cardiotonic steroids, faecal material was collected as described in the previous section, and experimental work was conducted at the laboratories at the Department of Pharmacology (University of Pretoria). Rat faecal material (1 g) was freeze-dried and sonicated for 15 min, followed by vortexing for one hour in solvent, either butanol or methanol (10 ml). The samples were clarified by centrifugation (3200xg; 10 min), and the organic phase was dried under nitrogen at ambient temperature. Extracts were reconstituted in methanol/water (1:3 v/v; 1 ml), containing 0.5 ng digoxin-d3/ml, and loaded onto the C18-reverse phase SPE cartridges (Strata, Phenomenex, Torrance, California, USA) preconditioned with methanol/water (v/v) and equilibrated with 10 mM ammonium acetate (pH 6.4; 1 ml). After loading the samples and washing with 10 mM ammonium acetate/methanol (7:3 v/v; 1 ml), the cardiotonic steroid analytes were eluted with chloroform/isopropanol (9:1 v/v) into 2 ml Eppendorf tubes. The wash and eluted fractions were dried and stored at −80 °C

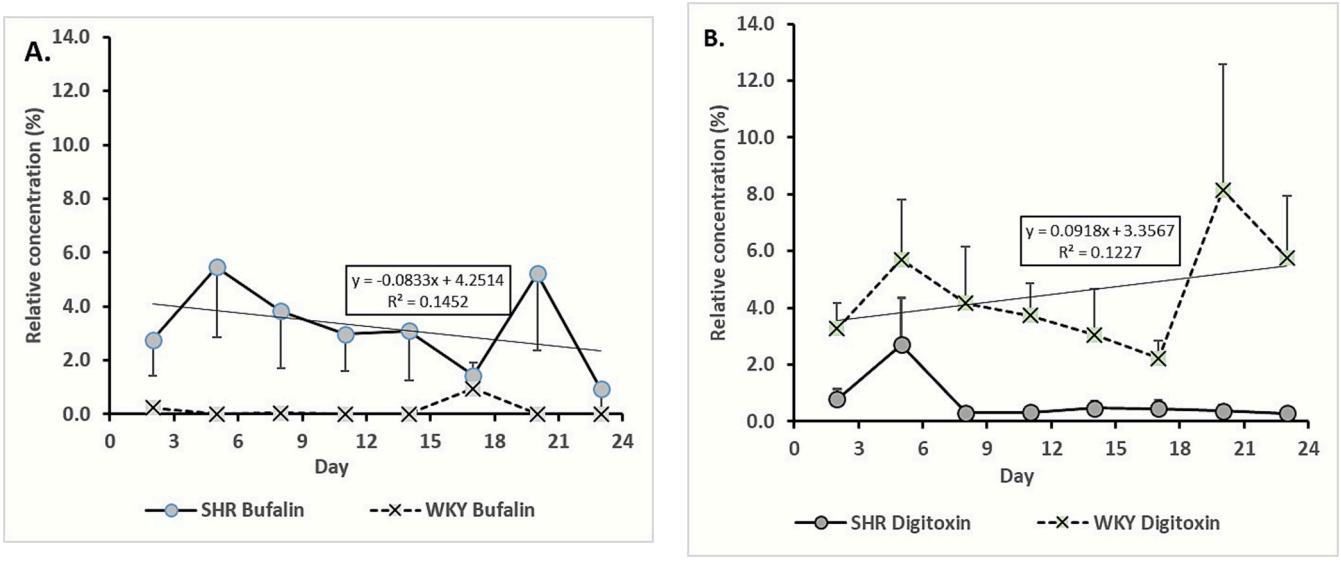

Fig. 2. Time course of A) bufalin and B) digitoxin ratios in SHR and WKY rats, respectively, over the 24-day experiment. Cardiotonic steroids (CTS) peak areas were expressed relative to detected bile acids (CTS peak area)/(LCA + HDCA + CTS peak areas) to correct for dilution. A. Bufalin was detected in most SHR faecal samples and in few WKY rat faecal samples, and vice versa (B.). Abbreviations: SHR: spontaneously hypertensive rats; WKY: Wistar Kyoto rats. HDCA: hyodeoxycholic acid; LCA: lithocholic acid.

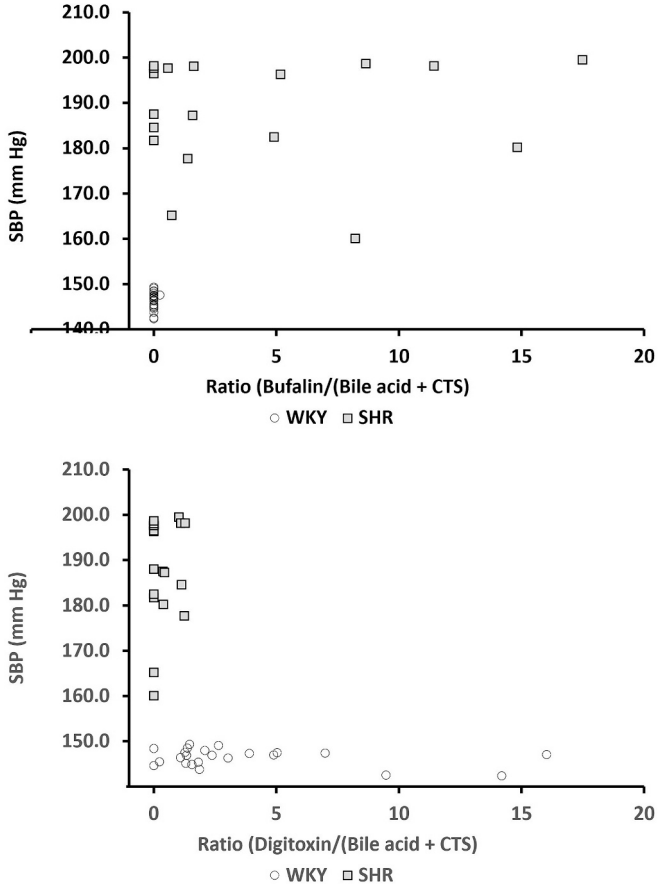

Fig. 3. Scattergram of the bufalin (top) and digitoxin (bottom) ratios with systolic blood pressure (SBP) in SHR and WKY rats. Cardiotonic steroids (CTS) peak areas were expressed relative to bile acids measured (CTS peak area)/(LCA + HDCA + CTS peak areas) to correct for dilution. Abbreviations: SHR: spontaneously hypertensive rats; WKY: Wistar Kyoto rats.

until analysed. Similarly, human faecal material (1g) was freeze-dried

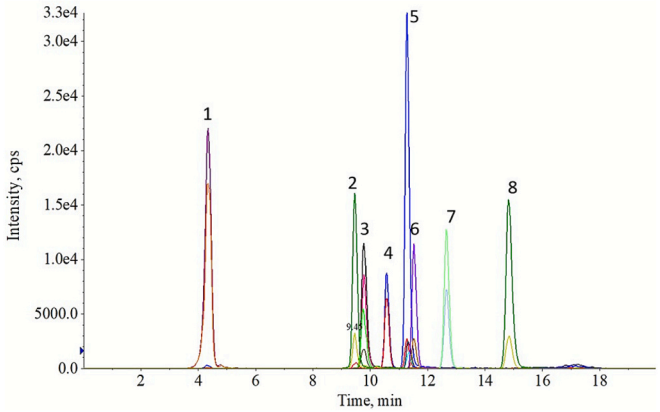

Fig. 4. Chromatogram of eight cardiotonic steroid standards separated on a Kinetex C18, 100 x 4.6 mm, 2.6 μm column with mobile phases (see text) A: 0.1 % formic acid in water and B: 0.1 % formic acid in acetonitrile using the gradient shown in Table S1.1. Ouabain (1; 4.3 min), periplogenin (2; 9.4 min), digoxin/digoxin-d3 (3; 9.8 min), telocinobufagin (4; 10.6 min), digitoxigenin (5; 11.3 min), marinobufagin (6; 11.6 min), bufalin (7; 12.7 min), digitoxin (8; 14.9 min).

and cardiotonic steroids were extracted with either MTBE, methanol, methanol:chloroform (1/2 v/v), or isopropanol (IPA):chloroform (v/v).

2.3.5. HPLC-MS analysis

Cardiotonic steroids extracted from the samples were separated on a Phenomenex™ Kinetex C18 reverse phase column (2.6 μm, 100 mm × 4.6 mm; Torrance, California, USA) using the final HPLC gradient summarised in Supplementary Table S3. An Agilent 1100/1200 combined series HPLC system (Agilent Technologies, Waldbronn, Germany) was coupled to an AB Sciex 4000 QTRAP triple quadruple tandem-mass spectrometer equipped with a Turbo V™ ESI source (Supplementary Table S4; Sciex, Toronto, Canada). System settings, analyte detection parameter optimisation, data acquisition and analysis were managed by Analyst® Software version 1.5.2 (Sciex, Toronto, Canada).

Standards were diluted in acetonitrile containing 5 mM ammonium formate and injected directly into the ESI source using a Harvard syringe

pump at a flow rate of 20 μL/ min. The masses of the analytes of interest were determined using a Q1 + scan. The declustering potential and collision energies were optimised using the parameter-ramp functions to produce the most intense precursor ion pairs for all analytes. A multiple reaction monitoring (MRM) algorithm was used to optimise selectivity and sensitivity during mass spectrometric analysis. Prepared samples (5 μL) were injected onto the column and run following the MS conditions optimised for the cardiotonic steroids (Supplementary Tables S3 and S4). Analyte linearity, selectivity, accuracy, precision, absolute and relative recoveries, detection and quantitation limits, reproducibility, matrix effects, and stability were determined (Supplementary Tables S5, S6).

2.3.6. Data and statistical analysis

Analyst software version 1.5.2 (AB SCIEX and Applied Biosystems/ MDS Analytical Technologies; Danaher Corporation, USA) was used to process the relative intensities and concentrations of cardiotonic steroids derived from mass spectrometric analysis and cardiotonic steroid recoveries, precision and linearity of the methods were determined. Statistical differences between rat groups and interventions were determined using ANOVA and Kruskal-Wallis test using GraphPad Prism 8 and Statistica with a p-value of less than 0.05 considered significant.

3. Results

3.1. Changes in cardiotonic steroids over time

The peak areas of the two positively identified cardiotonic steroids bufalin and digitoxin, measured relative to bile acids by HPLC/MS (Section 2.3.3.) showed the proportions of bufalin and digitoxin did not change significantly over the 24 days of the experiment in either SHR (bufalin: $p = 0.78$; digitoxin: $p = 0.71$) or WKY rats (bufalin: $p = 0.66$; digitoxin: $p = 0.26$) (Fig. 2A and Fig. 2B).

3.2. Associations of cardiotonic steroids with blood pressure in SHR and WKY rats

Significant differences in the ratios of bufalin and digitoxin were noted between WKY and SHR strains but neither steroid was associated with tail-cuff systolic blood pressure in either strain. Bufalin was not detected in most WKY rat samples, but when present, it was higher in the SHR samples ($p < 0.05$). Conversely, digitoxin was detected in all WKY samples and was absent in most SHR rats (Fig. 3). There appeared to be no association between the ratio of the detected cardiotonic steroids and sex in either rat strain (data not shown).

3.3. LC-MS method development and validation

When optimising the separation of cardiotonic steroids, both a Kinetex C18, 100 x 4.6 mm, 2.6 μm column with mobile phases A: 0.1 % formic acid in water and B: 0.1 % formic acid in acetonitrile, and a Kinetex Biphenyl column 100 x 4.6 mm, 2.6 μm column, with mobile phases A: 5 mM ammonium formate in water, and B: 5 mM ammonium formate in 90 % acetonitrile were compared. Superior separation was achieved with the C18 reverse phase column and mobile phases buffered with ammonium formate (Fig. 4). Positive ionisation mode provided higher sensitivity to detect cardiotonic steroid compounds with either $[M + H]^+$, ammonium $[M + NH_4]^+$ or sodium adduct ions $[M + Na]^+$ selected as these generated the highest intensity, the most stable ions with favourable fragmentation [45] (Table 1; Fig. S1-3).

Periplogenin and telocinobufagin in human faecal material eluted from the HPLC column as more than one peak. The periplogenin standard eluted as a single peak at 9.2 min. In contrast, in all the samples, it eluted as two peaks with retention times at 9.1 and 9.7 min, respectively, under the conditions used (Fig. S4). The first eluting peak was the high-intensity peak, whereas the second peak had the highest intensity with samples extracted with MTBE. Similarly, multiple peaks were identified with telocinobufagin with the standard retention time of 10.2 min and two other peaks at 9.5 and 10.7 min, which might be isomers with the same mass and similar fragmentation patterns as telocinobufagin. Hence, in standards only the peak at the same retention time (10.2 min) as the prepared standard was considered.

Calibration curves (6 points) covered ranges between 0.1–80 ng/mL with a linearity of $r^2 > 0.99$. LOD ranged from 0.01-0.5 ng/ml and LLOQ < 2 ng/ml depending on the cardiotonic steroid. Accuracy and precision were assessed and found to have a CV ≤ 20 %, except for digoxin (<26.3 %) and marinobufagin (26.7 %) and with an accuracy 75 % (digitoxin) −130 % (telocinobufagin) Extraction recovery was calculated as Recovery (%) = 100 × (measured concentration in extracted spiked sample − endogenous)/(concentration spiked into matrix) was > 80 % except for digitoxigenin. Matrix effects were evaluated by post-extraction spike: Matrix effect (%) = 100 x (peak area in post-

Table 1
Optimised respective MS detection parameters for precursor and product ions for cardiotonic steroid standards. DP: declustering voltage; CE: collision energy.

Analyte and Chemical Formula	Precursor ions			Product ions (*m/z*)	Fragmentation	CE (V)
	Monoiso-topic Mass (g/mol)	$[M + H]^+$/ $[M + NH_4]^+$	DP (V)			
Bufalin $C_{24}H_{34}O_4$	386.532	387.3 (H^+)	98	351.4	$[M + H\text{-}2H_2O]^+$	31
				145.2		51
D3-digoxin $C_{41}H_{64}O_{14}$	783.967	801.6 (NH_4^+)	61	654.3	[a]$[M + H\text{-}D]^+$	15
				97.4	[a]$[D + H\text{-}H_2O\text{-}OH]^+$	75
Digitoxigenin $C_{23}H_{34}O_4$	374.521	392.5 (NH_4^+)	83	339.2	$[M + H\text{-}2H_2O]^+$	25
				90.9		96
Digitoxin $C_{41}H_{64}O_{13}$	764.900	782.6 (NH_4^+)	54	635.2	[a]$[M + H\text{-}D]^+$	16
				113.3	[a]$[D + H\text{-}H_2O]^+$	65
Digoxin $C_{41}H_{64}O_{14}$	780.949	798.5 (NH_4^+)	57	651.7	[a]$[M + H\text{-}D]^+$	19
				97.4	[a]$[D + H\text{-}H_2O\text{-}OH]^+$	68
Marinobufagin $C_{24}H_{32}O_5$	400.225	401.6 (H^+)	78	365.2	$[M + H\text{-}2H_2O]^+$	26
				159.5		43
Ouabain $C_{29}H_{44}O_{12}$	584.652	585.7 (H^+)	56	385.0	[b]$[M + H\text{-}R\text{-}2H_2O]^+$	26
				403.4	$[M + H\text{-}R\text{-}H_2O]^+$	19
Periplogenin $C_{23}H_{34}O_5$	390.24	408.5 (NH_4^+)	45	337.3	$[M + H\text{-}3H_2O]^+$	26
				124.9		50
Proscillaridin A $C_{30}H_{42}O_8$	530.650	531.5 (H^+)	126	84.0		65
				513.4	$[M + H\text{-}H_2O]^+$	27
Telocinobufagin $C_{24}H_{34}O_5$	402.24	403.6 (H^+)	78	384.9	$[M + H\text{-}2H_2O]^+$	31
				171.3		51

[a] D = digitoxose (130 g/mol); [b]R = rhamnose (164.16 g/mol).

extraction matrix / peak area in solvent) ranged from −35.3 % (bufalin) to 9.6 % for ouabain (Supplementary Tables S5 and Table S6).

3.4. Cardiotonic steroids in rat strains and impact of interventions

Bufalin, digitoxigenin, digitoxin, digoxin and marinobufagin were detected in all samples; however, telocinobufagin was not detected in Wistar and SHR control rats (Fig. 5). Marinobufagin was the most abundant cardiotonic steroid detected in human and rat samples. Compared to the control rats, digoxin ($p < 0.05$) and marinobufagin ($p < 0.01$) were higher in DSS rats when salt was provided in their drinking water. Of note was that ouabain, proscillaridin A and periplogenin were not detected using the extraction methods (Table S7) as ouabain was not retained on the RP-SPE cartridge.

Overall, these data show multiple cardiotonic steroids were detectable in faecal material from untreated rats and humans at concentrations below that of the standard curves which varied with rat strain, and increased concentrations of digoxin were apparent with salt intervention in the DSS rats.

3.5. Extraction of cardiotonic steroids from human faecal samples

Significantly different amounts of digoxin, digitoxin, and telocinobufagin were extracted with different solvents. Isopropanol/chloroform and methanol/chloroform mixtures extracted more aglycone bufadienolides, with methanol extracting more cardenolide glycosides (Fig. 6 and Table 2). However, MTBE was less efficient at extracting these cardiotonic steroids.

4. Discussion

The main findings of this study determining unconjugated cardiotonic steroids in faeces of untreated rats and humans were a) the number of cardiotonic steroids detected simultaneously included the cardenolides: digitoxin, digoxin, digitoxigenin, periplogenin, and bufadienolides: marinobufagin, bufalin and telocinobufagin; b) solvent composition was critical in determining the selection of cardiotonic steroids extracted; c) cardiotonic steroids were identified in most faecal samples however, some cardenolides, notably ouabain, were not detected using the extraction methods and d) there were significant differences in cardiotonic steroid concentrations between rat strains and interventions; e) marinobufagin appeared to be the most abundant of the steroids detected; f) although the ratio of two steroids, bufalin and digitoxin, differed markedly between SHR and WKY rats, these ratios remained relatively constant over 24 days. These appear to be novel findings, and the presence of digoxin and digitoxin in this biological

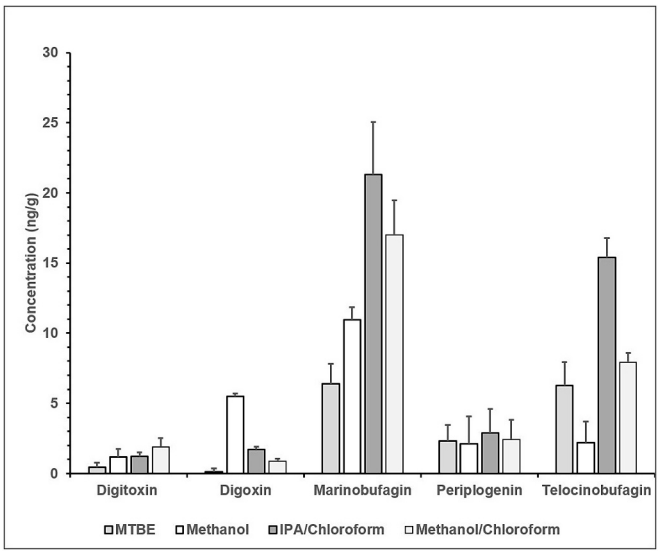

Fig. 6. CTSs extraction from spiked human faecal samples with different solvents. MTBE (n = 8); methanol, isopropanol (IPA)/chloroform & methanol/chloroform (n = 6). Statistical differences are shown in Table 2.

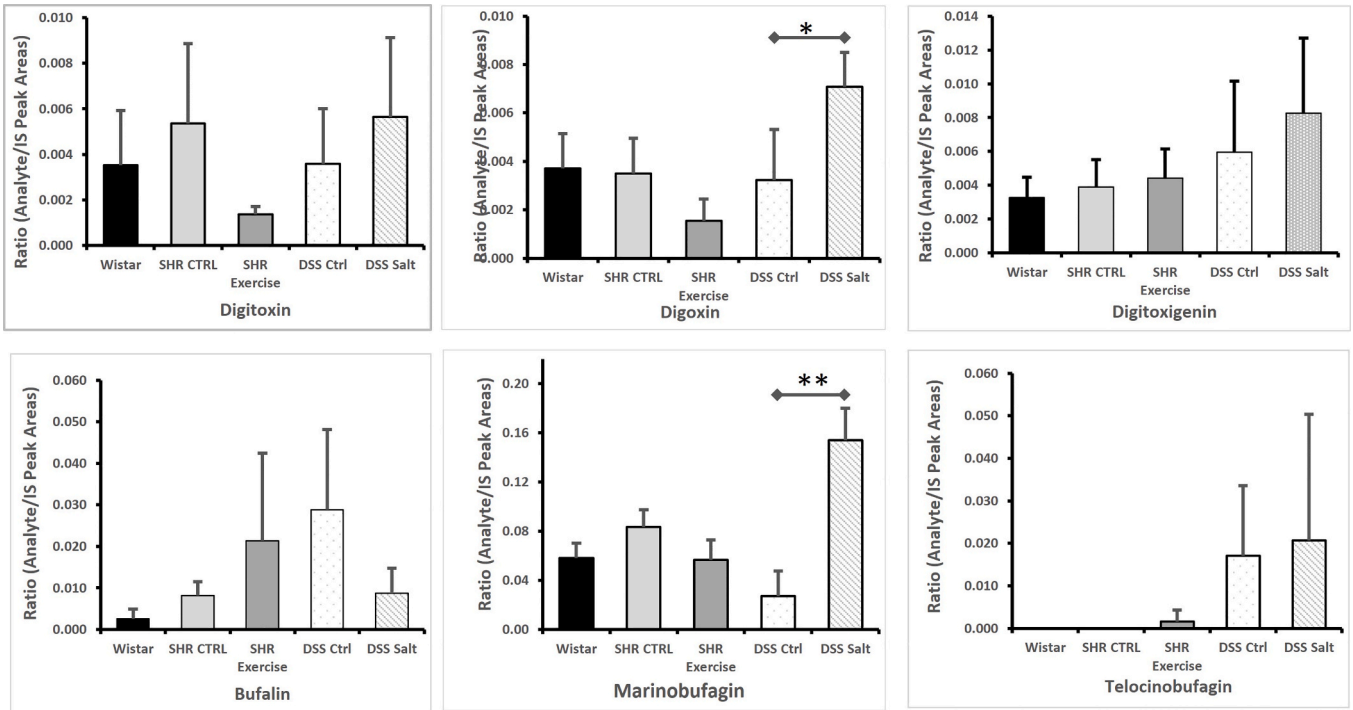

Fig. 5. Comparative amounts of cardiotonic steroids detected in different rat faecal samples. Data was reported relative to the internal standard as concentrations were less than the lowest standard. Wistar and SHR control (n = 9); SHR treated with exercise and DSS treated with salt (n = 6); DSS control (n = 18). Statistical significance (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). See Table S7 in Supplementary Tables.

Table 2
Statistical differences (see Fig. 6) of CTS concentrations extracted by different solvents (first row) relative to the solvent indicated in the second row. Abbreviations: $CHCl_3$ – chloroform; IPA – isopropanol; MeOH – methanol; MTBE – methyl-tert-butyl ether. Statistical significance of change relative to the comparative solvent: ↑ increased or ↓ decreased ns: not significant, $p > 0.05$; **$p \leq 0.01$, ***$p \leq 0.001$).

Extraction solvent	MTBE	MTBE	MTBE	MeOH	MeOH	IPA/ $CHCl_3$
Comparative solvent	MeOH	IPA/ $CHCl_3$	MeOH/ $CHCl_3$	IPA/ $CHCl_3$	MeOH/ $CHCl_3$	MeOH/ $CHCl_3$
Digitoxin	↓ **	↓***	↓***	*ns*	*ns*	*ns*
Digoxin	↓***	↓ **	↓ **	*ns*	↓***	*ns*
Marinobufagin	*ns*	*ns*	↓ **	*ns*	*ns*	*ns*
Periplogenin	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Telocinobufagin	*ns*	↓**	↓***	*ns*	↓***	↓***

matrix from these untreated animals or humans has apparently not previously been reported.

Excretion of digitoxin and digoxin in bile and by P-glycoprotein [24] explains the presence of these steroids in faecal material as both steroids have similar structures and undergo entero-hepatobiliary circulation [24,27]. Digitoxin was identified predominantly in WKY rats with lower blood pressure (Fig. 3). Digoxin does not increase blood pressure in normal rats and both digoxin and digitoxin effectively reduced ouabain-induced blood pressure [46]. Furthermore, digoxin has been shown to lower blood pressure in humans with hypertension [47], with therapeutic doses of digoxin and digitoxin decreasing 24-hour ambulatory nocturnal diastolic blood pressure and heart rate in normotensive subjects [48].

In contrast, bufalin was detected almost exclusively in SHRs with significantly higher blood pressure than WKY rats (Fig. 3). The finding of bufalin in faecal material would be consistent with endogenous synthesis and enterohepatic circulation, and with reports of bufalin immunoreactivity in the bile of untreated humans of 27.5 ng bufalin equivalents/ml whereas digoxin concentrations were below the detection limit (<0.5 ng digoxin equivalents/ml) [37]. Furthermore, bufalin increased heart rate, urinary output, and sodium excretion in a dose-dependent manner in rats and significantly raised blood pressure with either acute or chronic administration of bufalin [49]. Thus, the findings of higher digitoxin in WKY and bufalin SHR rats appear to align with the reported effects these two cardiotonic steroids in the literature. Whether or not these steroids directly contribute to the observed blood pressure phenotype in these rats, and the origin of digoxin and digitoxin in faecal material will require further investigation. The finding of raised marinobufagenin in DSS rats in the presence of salt in their diet is in keeping with the literature [12]. However, the finding of elevated digoxin with salt in the DSS rats is unexpected and might be explained by genetic differences in this strain [50].

4.1. Methodological and analytical aspects

Investigations of cardiotonic steroids may be broadly categorised by those determining concentrations associated with disease states and processes [4,6], those analysing profiles present in toad parotid gland secretions [51], and pharmacokinetic studies of Eastern traditional medicines [52–55]. Analytical methods for determining oleandrin [56], marinobufagenin [12,57] and for monitoring the drug digoxin [58] have been reviewed. Complex biological matrices may contain multiple cardiotonic steroids and metabolite isomers of identical molecular weight [45] and immunoassays are limited by antibody cross-reactivity. Accurate identification of these steroids requires optimal extraction, chromatographic separation and detailed structural elucidation. Such identification is facilitated by concentration steps, as low levels of cardiotonic steroids are present in biological samples. We therefore used readily available faecal material in which we hypothesized these steroids might be present in concentrations that were greater than that present in urine or plasma.

The present study concentrated faecal samples by freeze-drying, which was previously shown to yield good recoveries of unconjugated bufadienolides from toad venom [59]. Drying such samples under vacuum at 60 °C efficiently recovered bufadienolides and their conjugated derivatives, whereas prolonged air drying at room temperature resulted in a substantial loss of these compounds [59,60].

The present study extracted multiple free cardiac glycosides from faecal material of untreated rats and humans, with recovery depending on the extraction solvent used. The present study initially used MTBE, a solvent used for high throughput lipidomics [40] and the extraction marinobufagenin from tissues [39]. However, MTBE appears to be more efficient for the extraction of phospholipids, ceramides and related lipids [40].

Cardiotonic steroids have been extracted from plasma and urine by solid-phase extraction and eluted with methanol [3], and other solvents [61]. Methanol has been widely used to extract bufadienolides, metabolites and conjugated derivatives from toad parotid glands, traditional Chinese medicines [10,11,62], and plasma following oral administration [28,29,33,63,64]. Ethyl acetate extracted these steroids from plasma, tissues [30–38,41] and bile [34–36], orally administered oleandrin from mouse plasma, homogenised tissues and faecal matter [65] and as a general solvent to extract genins from plants [66]. The improved extraction of these steroids with solvent mixtures containing chloroform is consistent with reports of enhanced recovery of bufadienolides from plant and animal tissues [66,67], and of cardenolides from plasma [45]. Sequential extraction with solvents of different polarities (methanol, followed by heptane and then dichloromethane) improved recovery of bufadienolides from toad skin [68]. Furthermore, the suggestion that extraction of cardenolides from plants by removing fats with ether, steroid-binding lignin, which would be present in faecal material, and then sequential extraction with solvents of various polarities [69], may be an approach when extracting cardiotonic steroids from faecal material. The non-detection of particular cardiotonic steroids, such as ouabain, in this and other studies [70], can be explained by differences in solid-phase extraction media [3], solvents and other experimental methods.

Separation and identification of cardiotonic steroids was achieved using a C18-reversed column and mobile phases buffered with ammonium formate. Solvent selection affects the intensity of fragments [71] and the use of d3-digoxin [72] aided identification of standards. Reporting recovery of cardiotonic steroids from spiked biological fluids is limited as such matrices contain these steroids [60]. The finding of telocinobufagin and periplogenin standards yielding multiple chromatographic peaks has been previously reported [45] and fragmentation of cardiotonic steroids by MS with the loss of water and the glycosidic moiety is consistent with other studies [61,71]. The lack of tandem MS/MS and analytical standards to identify additional cardiotonic steroids and their metabolites was a limitation of this study. Tandem MS/MS improves identification [55] with a report of two-dimensional separation with normal phase and reversed phase HPLC columns and MS/MS determining 64 bufadienolides and derivatives from toad skin extracts [73]. Similarly, HPLC-ESI-Q-TOF-MS/MS profiled and identified 54 bufogenins and 63 bufotoxins from toad venom extracts of a traditional Chinese medicine (Chansu) [74].

We hypothesised that faecal concentrations of cardiotonic steroids might be higher than in urine and plasma. However, these steroids were only detected at concentrations below the minimum concentrations of prepared standards and were therefore reported as intensity or relative to bile acid peak areas to account for dilution effects. As noted, cardiotonic steroids are synthesised from bile acid intermediates [41,42], and as bile acid turnover is low and tightly controlled [44], the ratio of the steroid peak area relative to that of steroid peak areas plus those of lithocholic acid and hyodeoxycholic acid, the predominant and detected bile acids in rat faecal material [75,76] appeared to be

appropriate to correct for dilution. Shafaei et al. [77] reported bile acids relative to faecal wet weight as extraction of these bile acids from dried faecal material was variable, reflecting the non-homogeneity of faecal material. Despite such limitations, the study found the ratio of extracted digitoxin in WKY rats and bufalin in SHRs, relative to abundant faecal bile acids detected did not vary substantially over 24 days the rat models.

Variation in binding and inhibition constants of cardiotonic steroids for Na/K-ATPase [78,79] suggests cardiotonic steroids of higher affinity may replace those of lower affinity from the binding site [80] with associated physiological changes. Indeed, antagonism between cardiotonic steroids [81] such as the digitoxigenin analogue, rostafuroxin, displaced ouabain from the Na/K-ATPase binding site to reduce blood pressure [82]. Similarly, spirolactone competitively replaced ouabain to reduce collagen formation in cardiac tissue [83]. This may suggest that changes in cardiotonic steroid profiles occur over time and may explain the phenotypic changes that occur as a disease progresses.

4.2. Pharmacokinetics and implications

Cardiotonic steroids are produced endogenously in the body of untreated animals and humans [4,6,8,9] and their elimination is by the kidney, and importantly, the gastrointestinal tract in bile [34–36] and excretion by P-glycoprotein [24]. Indeed, transport by P-glycoprotein plays a significant role in regulating plasma concentrations of digoxin [84]. The pharmacokinetics of cardiotonic steroids explain many of the findings reported in this study.

Cardiotonic steroids can be synthesised from bile acids [41,42] and both are modified by the gut microbiome. The microbiome affects the availability digoxin [85], antihypertensive and other drugs [86]. Bile acids, upon re-circulation, are converted to secondary bile acids and metabolites by the gut microbiome [87,88]. Similarly, bacteria and fungi are capable of catalyzing an array of transformation reactions to alter and metabolize cardiotonic steroids [89,90].

Dysbiosis of the gut microbiome is associated with a host of diseases [91] including cardiovascular and cardiometabolic diseases [92], such as preeclampsia [93], myocardial infarction [94], heart failure [95] and the underlying hypertension [96,97], all conditions associated with known alterations in cardiotonic steroid concentrations [4,6]. The microbiome and bile acids are also altered in cardiometabolic diseases [98] and such associations require further investigation.

Although changes in the microbiome occur in cancers [99], concentrations of associated cardiotonic steroids have not been specifically studied. Cardiotonic steroids have emerged as important lead compounds for inhibiting P-glycoprotein and overcoming cancer drug resistance [100]. However, despite promising pre-clinical data, a *meta*-analysis of clinical trials concluded no benefit of these steroids in treating or reducing the recurrence of cancer [101]. However, such studies have been limited to commercially available cardiotonic steroids and knowledge of cardiotonic steroid profiles in cancers with disease progression may provide useful information to guide potential therapeutic interventions.

The finding of multiple cardiotonic steroids in faecal material will allow studies to determine associations between the microbiome and cardiotonic steroid profiles, transformations to metabolites and changes over time with concurrent changes in the intestinal microbiome. This may provide interesting mechanistic insight into the development and progression of certain diseases.

5. Conclusions

The study identified multiple cardiotonic steroids present in the faecal material of untreated rats and humans. Particular steroids were associated with certain rat strains and marinobufagin was predominant. Bufalin was identified in most SHR rats and digoxin in almost all WKY rats. Digitoxin increased with salt in DSS rats.

The critical step to determine the cardiotonic steroid profile present in biological samples is the reliable extraction of these steroids as no single solvent or solvent mixture appears to fully extract these from biological samples. The approach of removing interfering non-steroidal lipids, using SPE cartridges and the sequential extraction with solvents of different polarities appears to be an approach when determining the profile of cardiotonic steroids present. The analysis of faecal material is a non-invasive means of obtaining biological samples in reasonable quantity for studies of these steroids.

CRediT authorship contribution statement

Zelie F. Masso: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation. **Hannah Bint Ebrahim Mullah:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Anza Thiba:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Sarhana Dinat:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Ekene E. Nweke:** Supervision, Writing – review & editing. **Gavin R. Norton:** Methodology, Conceptualization. **Angela J. Woodiwiss:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **A. Duncan Cromarty:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Geoffrey P. Candy:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data is available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2026.109747.

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