Recent Advances in Hypertension

Endogenous Ouabain
Recent Advances and Controversies

John M. Hamlyn, Mordecai P. Blaustein

In this brief article, we summarize recent reports about endogenous ouabain (EO), a cardiotonic steroid hormone. This includes analysis of mammalian EO, the discovery of EO isomers, regulation of intracellular signaling by EO, and the roles of EO in hypertension, pregnancy, and heart and kidney diseases. Novel ouabain-resistant mice that elucidate the key roles of α2 Na⁺ pumps and their cardiotonic steroid-binding site are also discussed.

EO and Its Isomers
EO was first identified in human plasma 25 years ago.1,2 Despite confirmation in humans and other mammals with mass spectrometry (MS; Figure; Figures S1–S6 in the online-only Data Supplement), nuclear magnetic resonance, and combined liquid chromatography (LC)–immunology methods,3–6 only Data Supplement), nuclear magnetic resonance, and combined liquid chromatography (LC)–immunology methods,3–6 human EO has remained controversial.7 New analytic studies and related findings should allay skepticism. For example, employment of multistage MS (MS–MS and MS–MS–MS) to examine the effects of pregnancy and of central angiotensin (Ang) II infusion on EO in rat plasma led to the discovery of 2 novel EO isomers.8,9 Isomer 1 has MS–MS and MS–MS–MS product ion spectra indistinguishable from those of EO, but is slightly more polar than EO; it binds to the antibody used in our radioimmunoassay. Isomer 2 is slightly less polar than EO, has a distinct MS–MS–MS spectrum, and cross reacts weakly in our radioimmunoassay. The primary structural difference(s) between EO and these isomers may involve the steroid nucleus. Importantly, neither isomer was previously described or is detectable in commercial (plant) ouabain.8,9

A recent report based on an LC–MS–MS approach concluded that EO was not detected in human plasma,10 but the LC gradient was extraordinarily short so that EO in plasma may have been missed (Data Supplement). Furthermore, critical data supporting their conclusion were absent from the published article,10 and the key product ion current recording had inexplicable gaps (Figure S7) at locations where signals from EO isomers might be anticipated.11 Also, the plasma used by Baecher et al10 tested positive for EO11 with a well-documented radioimmunoassay.8,12 These radioimmunoassay data are significant because EO is routinely detected when the same sample extracts are subjected to LC-radioimmunoassay and LC–MS (Figure).5,8,9,12 In contrast to MS, radioimmunoassay-based estimation of EO includes the unpredictable contribution of cross-reactivity from related molecules,5,13 such as isomers 1 and 2,8,9 which may vary with sex, age, and disease. The carbon isotope ratio (13C/12C) is helpful to distinguish plant versus animal metabolism. The natural abundance of 13C in the bovine adrenal EO and, thus, the 13C/12C ratio determined by high-resolution MS was significantly lower than in plant ouabain.10 Therefore, EO is neither a laboratory contaminant nor an ingested plant material. If adrenal EO is not plant ouabain seques tered from the circulation,8,9 it must be, either in whole (ie, sugar and steroid) or in part (steroid, alone), an endogenous product.

What Is the Origin of Circulating EO?
Human, bovine, and rodent data indicate that the adrenal cortex contains the highest concentration of EO in the body.1,11 Also, adrenalec tomized rats1 and adrenal insufficiency patients16 have exceptionally low plasma EO levels. Primary cultured bovine and human adrenal cortical cells secrete more EO than is present in the cells, indicating net synthesis.17 Adrenal venous EO concentrations (adrenal vein cannulation) in the dog were 4- to 5-fold higher than that in arterial blood.18 Similarly, in human hypertensives undergoing testing for hyperaldosteronism, the adrenal venous effluent EO concentration was 2- to 3-fold higher than in inferior vena cava blood.19 In that study, MS–MS–MS analysis of the plasma confirmed that the endogenous substance was EO and an isomer (likely isomer 2). Thus, the adrenal cortex is most probably the primary source of circulating EO, and aldosterone and EO biosynthesis share a requirement for progesterone.20 The brain is likely also a source of 1 isomer.9 Regrettably, the biosynthetic pathway for EO remains unresolved. This is, in part, because of the difficulty and the resources required to elucidate an adrenal pathway whose relative carbon flux is ≈20- to 50-fold and ≈10,000-fold less than for aldosterone and cortisol, respectively.

Role of the Brain in Regulating Circulating EO
Early work suggested that the central nervous system (CNS) influences the peripheral levels of ouabain-like substances.21 Indeed, brain ouabain-like materials are critical to the ability of low-dose angiotensin (Ang) II to raise circulating EO and blood pressure.

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526
EO receptor site on α2 Na+ pumps to make these pumps ouabain-resistant (α2Oo) blocks ouabain-induced and salt-sensitive forms of hypertension in mice. Importantly, pressure overload-induced cardiac hypertrophy and failure are greatly attenuated in α2Oo mice, whereas they are accelerated in these mice when the α1 Na+ pumps are mutated to an ouabain-sensitive form. (note: the α1:α2 expression ratio is ≈4:1 in heart and arteries.) Thus, in addition to hypertension, target organ damage depends, in part, on high-affinity EO binding (see EO in Kidney Disease and Heart Failure section of this article).

**EO Is Part of a New Neurohumoral Pathway in BP Control**

Compelling evidence indicates that the slow pressor effects of low doses of Ang II depend on an amplifier located in the CNS. The amplifier incorporates neuremodulatory components including local aldosterone synthesis, mineralocorticoid receptors, ENaCs, and increased synthesis or levels of EO in the brain. Prolonged stimulation of this CNS amplifier, especially by Na+ or low-dose Ang II, increases sympathetic nerve activity, often to discrete vascular beds. In addition, however, activation of the CNS amplifier raises the circulating levels of peptide hormones including adrenocorticotropic hormone, a stimulator of adrenal EO secretion, vasopressin, and growth hormone. The relative roles of increased sympathetic nerve activity and the humoral components are not clear.

Intracerebroventricular Ang II infusion also elevates circulating EO. Sustained increases in circulating EO, per se, augment the expression of proteins involved in Ca2+ homeostasis and signaling in arterial myocytes. The effects of the elevated circulating EO on Ca2+ handling in arterial myocytes in vivo are fully replicated ex vivo with nanomolar ouabain. Notably, all the effects of intracerebroventricular Ang II on circulating EO, as well as the reprogramming of peripheral vascular function, and the elevated BP are prevented by intracerebroventricular administration of eplerenone, a mineralocorticoid receptor blocker, as well as by inhibition of aldosterone synthase with FAD286. Furthermore, BP elevation by subcutaneous low-dose Ang II plus high dietary salt is greatly attenuated by immunoneutralization of EO with fab fragments that bind ouabain with high-affinity. Apparently, EO itself can augment basal and stimulated vascular tone and raise BP.

The demonstration that brain Ang II activates a novel long-range neurohumoral–vascular control axis that involves EO is striking. This axis amplifies the long-term central effects of Ang II by recruiting CNS components (aldosterone, mineralocorticoid receptors, ENaCs, and brain EO) and peripheral factors that include circulating EO and upregulated expression of Ca2+ transport proteins in arterial myocytes. Collectively, these factors contribute to the ability of chronic central Ang II and increased sympathetic nerve activity to elevate Insert: BP and maintain high BP. We postulate that this CNS–humoral axis is part of the “other mechanism” that helps maintain the elevated BP when the direct vasopressor activity of chronically elevated circulating Ang II “plays only a minor role.”

**Na+ Pump Is A Biased Receptor for EO**

The physiological and pharmacological effects of the cardio- tonic steroid have long been interpreted as the consequence
of binding to a highly conserved site on the Na⁺ pump catalytic (α) subunit and the block of Na⁺ transport. This was confirmed by studies in α2R/R mice and mice lacking Na/Ca exchanger-1.

The groundbreaking observation that ouabain binding also activates signaling cascades added critically to the mounting evidence that ouabain is a hormone. The ouabain-stimulated signal transduction is mediated by Na⁺ pumps but is apparently independent of the ion transport function. Remarkably, recent work reveals that the ouabain-binding site behaves like a biased receptor, the first example of this phenomenon in an ion transport system. Ouabain binding to arterial Na⁺ pumps activates c-Src, for example, whereas the binding of digoxin, which is an equieffective pump inhibitor, does not. In fact, digoxin antagonizes ouabain’s effects and vice versa, both in vivo and in vitro. Thus, biased signaling likely underlies the ability of ouabain and EO to induce hypertension and explains both the inability of digoxin to raise BP and its antihypertensive effect in ouabain-dependent models.

Rostafuroxin (10 μg/kg per day), an ouabain antagonist, attenuates ouabain-induced hypertension in rats, but 5 mg/d was ineffective in unselected patients with stage I or II hypertension in the OASIS-HT (Ouabain and Adducin for Specific Intervention on Sodium in Hypertension Trial). Nevertheless, rostafuroxin effectively lowered BP in a sensitive cohort of patients with adducin variants and elevated blood pressure, suggesting that fluid volume in pregnancy is more relevant than previously appreciated: circulating volumes in women destined to become preeclamptic seem to be inappropriately elevated early in pregnancy. The mechanisms by which early volume overexpansion might trigger vascular changes that lead to preeclampsia require investigation.

Circulating ouabain-like materials rise progressively in normal pregnancy and decline after delivery. The earlier reports were recently confirmed with advanced analytical methods: in addition to circulating EO, one of the newly discovered isomers was markedly elevated in pregnancy. Based on the emerging pressor mechanism of ouabain, the elevated EO in pregnancy was expected to reprogram vascular function by increasing the expression of arterial myocyte Ca²⁺ transporters, for example, Na/Ca exchanger-1 and transient receptor potential channel-6. Uregulation of these proteins is triggered by the prolonged elevation of circulating ouabain in normal nonpregnant rats. In the high EO state of pregnancy, however, expression of Na/Ca exchanger-1, which mediates Ca²⁺ influx and tone in arterial myocytes, was minimal. In other words, normal pregnancy is a high EO state with apparent resistance of the arteries to the pressor action of circulating EO. Indeed, even supraphysiological circulating levels of ouabain failed to raise BP in pregnancy. The mechanism of ouabain resistance is likely to be significant in elucidating the decline of vascular reactivity in pregnancy. Nevertheless, the low BP in pregnant α2R/R mice indicates that the integrity of the α2 Na⁺ pump ouabain-binding site provides a small stimulus to BP in the third trimester of pregnancy.

Does elevated EO or EO resistance have any role in preeclampsia? Circulating EO is linearly related to BP in pregnancy, and reduced vasoreactivity in pregnancy, it is surprising that excess mineralocorticoid triggers a preeclampsia-like state in rats. Furthermore, excess antidiuretic hormone increase in early pregnancy may predict preeclampsia in humans. This suggests that fluid volume in pregnancy is more relevant than previously appreciated: circulating volumes in women destined to become preeclamptic seem to be inappropriately elevated early in pregnancy. The mechanisms by which early volume overexpansion might trigger vascular changes that lead to preeclampsia require investigation.

Role of Genetics in Ouabain-Induced Hypertension

Prolonged ouabain administration induces hypertension in many, but not all, outbred rodent strains. This variable response, even within a single strain, is neither strange nor surprising. When given high salt, excess mineralocorticoids or other hypertensinogenic substances, not all outbred rats develop hypertension; indeed, this phenotype variation was deliberately exploited to generate lines of rats with heightened or lowered susceptibility to hypertension. Experience with ouabain is no different. Starting from a large founding colony of outbred Sprague–Dawley rats in which high ouabain sensitivity was the dominant phenotype in both sexes, minimal inbreeding led to distinct strains with ouabain-sensitive and ouabain-resistant BP phenotypes within 3 generations. The sensitive strain exhibited altered ganglionic synaptic plasticity that was normalized with in vivo captopril. Some components of the pressor mechanism of ouabain that likely function in the sensitive strain have been partially elucidated, whereas elevated vagal tone and increased calcitonin gene-related peptide may underlie the ouabain-resistant phenotype.

Hypertension Mediated by Ouabain-Sensitive α2 Na⁺ Pumps in the Brain

Liddle syndrome is a salt-sensitive hypertension due to enhanced ENaC activity caused by loss of regulation by the ubiquitin ligase, NEDD4-2. A mouse model, the NEDD4-2 knockout (NEDD4-2−/−), with upregulated renal ENaCs, exhibits mild salt-sensitive hypertension. Brain ENaCs are also upregulated, and salt-sensitive hypertension is prevented by intracerebroventricular infusion of very low-dose benzamil, an ENaC blocker that inhibits the CNS neurohumoral pathway. Furthermore, although intracerebroventricular Na⁺-rich cerebrospinal fluid induces hypertension in wild-type and NEDD4-2−/− mice, the hypertension is prevented by expression of ouabain-resistant α2 pumps, that is, in α2R/R and NEDD4-2−/α2R/R mice. Thus, an EO-like compound and CNS, as well as renal, ENaCs, and α2 Na⁺ pumps, apparently participate in the hypertension of Liddle syndrome. This complements previous studies showing that α2 ouabain-binding site integrity and its ligand are essential for other forms of experimental hypertension.
EO could raise BP in a dose-dependent manner. Surprisingly, however, in pregnant rats with reduced uterine perfusion pressure and hypertension, prolonged exogenous ouabain administration (additional to the already elevated EO) lowered circulating sFLT1 (soluble fat mobilizing substance-like tyrosine kinase-1) and reduced BP.\textsuperscript{93} Thus, in this preeclamptic model in which EO is thought to be elevated, ouabain behaved as an antihypertensive and had a net effect on BP that resembled that of digoxin in ouabain-dependent hypertension. The mechanism of this paradoxical and beneficial effect requires investigation. Nevertheless, it now appears that, contrary to earlier ideas, EO upregulation in preeclampsia is of potential benefit to mother and fetus.

At the opposite end of the pregnancy spectrum, recent studies link low circulating EO levels with impaired fetal growth and development: In pregnant mice, antioouabain antibodies reduced circulating EO, decreased offspring body weight, and impaired kidney and liver growth. Furthermore, during human pregnancy, circulating EO among women with small-for-gestational age neonates was lower than in women with normal-for-gestational age newborns.\textsuperscript{92} Ouabain is recognized as a growth promoter, but these new results are the first to suggest that relative lack of EO increases the risk for impaired fetal development. In this context, the aforementioned ouabain resistance of pregnancy makes sense: the elevated circulating EO could exert a growth-promoting effect when the hypertensinogenic activity is deactivated. Further evidence that EO is a growth factor in pregnancy is that malnutrition delayed the formation of functional nephrons in the fetus and increased susceptibility to renal injury and disease later in life. Administration of ouabain to malnourished pregnant rats protected fetal kidney development.\textsuperscript{93}

**EO in Kidney Disease and Heart Failure**

Acute kidney injury is a frequent complication that increases the morbidity and mortality of cardiac surgery. EO can behave as an adrenal-derived stress hormone and has been associated with adverse cardiovascular outcomes in clinical studies. In data from 2 centers (626 patients), preoperative EO was the strongest predictor of surgery-induced acute kidney injury significantly improved predictability.\textsuperscript{94} Furthermore, a rat model of ouabain-induced hypertension exhibited reduced creatinine clearance, proteinuria, and impaired podocyte nephrin expression; thus, elevated EO per se may be a direct cause of podocyte damage.\textsuperscript{94}

EO, which may contribute to renal failure\textsuperscript{95} and may be linked to cardiomyopathy in chronic kidney disease,\textsuperscript{62,97,98} also seems to be a valuable biomarker of heart failure. In 845 patients undergoing elective cardiac surgery, plasma EO was correlated negatively with left ventricular ejection fraction, and positively with cardiac end-diastolic diameter and plasma N-terminal of the pro-brain natriuretic peptide. Higher EO levels immediately postoperatively were associated with increased 30-day perioperative mortality.\textsuperscript{99} Thus, both pre- and postoperative EO levels identify patients with more severe cardiovascular presentation and those with a higher risk of morbidity and mortality after cardiac surgery.\textsuperscript{99}

**Conclusion**

During the past 5 years, numerous notable advances have been made in the understanding of EO, its receptor and the downstream effects of activation of EO in the brain and periphery. Although many important questions remain to be investigated, compelling evidence indicates that EO is a significant entity in physiology and contributes to the pathogenesis of many common diseases.

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**Disclosures**

None.

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Mass Spectrometric Determination of EO

This supplement contains examples of LC-MS-MS determination of EO in human plasma (Figure S1; see Figure 1 in the main text for another example\(^1\)) and rat plasma (Figure S2) from our laboratory. Figure S3 shows the LC-MS-MS spectrum of exogenous (plant) ouabain for comparison. Figures S4 and S5 contain, respectively, multi-stage MS spectra of plasma EO from mice and rats with salt-sensitive hypertension. Figure S6 shows published LC-MS-MS spectra of human plasma EO from Komiyama et al.\(^2\)

In contrast, Figure S7 shows the LC-MS-MS spectra from a report by Baecher et al. that claims that EO is not detectable in human plasma.\(^3\) Not shown, are data from other groups that identified EO in plasma and tissue from rat and bovine sources.\(^4\)\(^-\)\(^6\) The LC-MS-MS examples in Figures S1-S4 and S6 all employed capillary solvent conditions in which the sample was injected into an LC column well pre-equilibrated with water. Following injection, the solvent gradient was allowed to develop slowly over 30 (Figure S6), 50 (Figures S2, S3, S4) or 80 minutes (Figure S1). Baecher et al.,\(^3\) however, used short “ballistic” LC gradients (<6 minutes; Figure 7S, in which the beginning of the LC run was not presented - compare Figure 1 of the main text and Figures S1 and S2). Like Baecher et al., we were unable to demonstrate EO (or added small spikes of commercial ouabain) in complex plasma extracts using short LC gradients and columns that were not well pre-equilibrated with water. In contrast, we readily detected EO in human and rodent plasma extracts by LC-MS-MS when we employed more favorable conditions (long LC gradients and LC columns that were extensively pre-equilibrated with water; see Figure 1 of the main text and Figures S1, S2 and S4-S6). Further, using even more advanced methods such as LC-MS-MS-MS, it was possible not only to quantitate EO, but to explore the significance of its two most prominent isomers.\(^7\)\(^,\)\(^8\)

In sum, the figures and reports mentioned here demonstrate that, with appropriate methods, EO can be identified by multi-dimensional MS in human, bovine and rodent plasma and tissues. The
inability of some workers to identify EO is apparently related to technical issues. Importantly, Figures S4 and S5 show that the plasma EO is influenced by dietary salt in salt-sensitive rodent models.
Figure S1. Sample LC-MS-MS of a plasma extract from a Milanese patient (#5) with untreated essential hypertension. The extracted plasma sample was separated by capillary HPLC (Agilent 1100) over 80 minutes using a slow acetonitrile gradient. The column eluate was mixed with lithium carbonate, and passed to the ESI interface on a Bruker (Billerica, MA) Esquire–LC ion trap mass spectrometer. The mixture was monitored continuously for lithiated molecular ions at 591 m/z (corresponding to the lithiated parent ion of EO; the diamond in “B”). Following collision-induced-dissociation (CID), Panel A shows the extracted ion current (EIC) chromatogram for lithiated molecular MS-MS product ions at 445 m/z (i.e., the aglycone of EO). A prominent product ion peak was detected at 53.2 min characteristic for the mobility of EO with the slow gradient LC gradient used. The spectrum of the MS-MS ion current (Panel B) shows the diagnostic fragment ions at 445.4 and 427.3 m/z which correspond, respectively, to the singly and doubly dehydrated derivatives of the Li-aglycone of ouabain. From Blaustein et al. Online Supplement\textsuperscript{9} with permission.
Figure S2. Endogenous Ouabain in Rat Plasma determined by LC-MS-MS. Fresh plasma from a normal male rat was extracted. 0.25 ml of the original plasma volume was separated by capillary (Zorbax C18, 0.3 x 150 mm) LC (Agilent 1100). In this case, a 50 minute gradient of acetonitrile was used to elute bound materials. The column eluate was monitored continuously for lithiated ion species over an abbreviated scan range (400-650 m/z) and selectively for positive ions equivalent to Li-ouabain (m+Li/z = 591.3). The top panel (red) shows the summed MS ion chromatogram for positive ions within the scanned range (400-650 m/z). The second panel (green) shows the extracted MS ion current chromatogram for positively charged molecular ions with m/z = 591.3 (i.e., equivalent to Li-EO). The third panel (blue) shows the extracted MS-MS ion current chromatogram resulting from the CID of all endogenous sample ions within the 591.3 m/z isolation window. Note the prominent MS-MS product ion peak eluting at 27.9 minutes. The MS-MS spectrum of this ion peak is shown in the bottom panel; molecular product ions at m/z 445.2, 427.2 and 409.2 (arrows) representing the Li-aglycone of EO and its two dehydrated derivatives, respectively, are readily apparent. Interpolation of the MS-MS ion current at 27.9 min with exogenous ouabain standards under identical conditions (Figure S3) showed
that the plasma EO content was ~141 pmoles/L. From Blaustein et al. Online Supplement with permission.

Figure S3. LC-MS-MS of Exogenous Ouabain. Following analysis of the rat sample in Figure S2 and washout of the column, exogenous ouabain (75 fmol) was injected. The elution conditions, mass spectrometer settings, and ion monitoring conditions were identical to those used in Figure S2. The top panel (red) shows the summed MS ion chromatogram for lithiated molecular ions within the scanned range (400-650 m/z). The second panel (green) shows the extracted MS ion current chromatogram for lithiated molecular ions with m/z = 591.3 (equivalent to Li-ouabain). The third panel (blue) shows the extracted MS-MS ion current chromatogram resulting from the CID of parent molecular ions at 591.3 m/z. A prominent MS-MS product ion current peak appeared at 27.9 minutes; the MS-MS spectrum of that ion peak (lower panel) shows a major product ion at 445.2 m+Li⁺/z, and minor ions at 427.2 and 409.2 (arrows), corresponding to the Li-aglycone of ouabain and its singly and doubly dehydrated
derivatives, respectively. The spectra are indistinguishable from those in Figures S1 (human plasma EO) and S2 (rat plasma EO). From Blaustein et al. Online Supplement with permission.
The high salt diet (4% NaCl, 14 days) raised (A) systolic BP and (B) plasma EO in the salt sensitive G protein-coupled receptor 4γ mutant (A486V) mice.

Intra-arterial catheter, n = 5-7; *P<0.05 vs normal salt (NS); tandem LC-MS-MS on pooled plasma samples.

**Figure S4. EO in GRK4-A486V Mice on a high salt diet.** Wild type and GRK4-γ mice were maintained on a normal (0.6%) or a high salt (4% NaCl) diet for two weeks by Dr. Pedro A. Jose. Intra-arterial systolic BPs were then measured and plasma was collected. After C18 extraction, 50 µl of plasma equivalents from each pool was injected into the LC-MS-MS (Bruker HCT Ultra) system using a slow gradient. The two top panels show molecular product ions of EO in the plasma from the wild type (left) and GRK4-A486V animals at 445.2 m/z with quantitation as in Figures S2 and S3; note the difference in the two ordinate scales. The lower left panel shows the characteristic molecular product ion (445 m+Li^+/z) for exogenous ouabain in the same system. The lower right panel shows the summary data for BP and EO. GRK4-A486V: G protein-coupled receptor kinase γ subunit with the A486V mutation found in some human EH. The GRK4-A486V mice are salt-sensitive due to defective dopamine signaling. Unpublished data of Hamlyn, Blaustein & Jose (2009).
Figure S5. EO in the Circulation of Dahl S and R Rats.
A. Sample Blank. B. 5 nM ouabain standard. C, D. Extracted plasma from Dahl salt-resistant rats (DR) and salt-sensitive rats (DS) on a 6% NaCl diet. DHO = dihydro-ouabain internal standard. Unpublished data from the same experiment as in Figure 31.2 of Pulina et al. (2013).12 The lithiated MS-MS-MS molecular product ions at 379.2 and 381.2 m/z are diagnostic for EO and DHO, respectively. After 11 days on 6% NaCl, DS and DR systolic BPs were 154±2 and 135±2 mm Hg (n=4 each), respectively, vs 113±2 and 120±2 mm Hg on normal salt. See Jacobs et al.8 and Hamlyn et al.7 for details of the MS-MS-MS (MS3) methods and examples of full spectra.
Figure S6. Analysis of EO in human plasma using negative ion LC-MS-MS. Left top panel shows the negative LC-MS ion chromatogram from 80 ml of pre-cleared human plasma (Waters Sep-Pak C18) applied to an LC-MS-MS system eluted with a slow acetonitrile gradient (0-50% in 30 min). The ion peak eluting at 20.1 min (m-H⁻/z = 583.1, identical to that of commercial ouabain) was further analyzed by MS-MS; chromatograms for selected ions with m-H⁻/z at 583.1 (EO), 437.2 (the aglycone of EO), and at 419.1 and 371.3 (its dehydrated fragments) are shown. The MS-MS product ion spectrum (right-hand panel) of the molecular ion eluting at 20.1 min shows fragments with mass to charge ratios that are consistent with those observed with commercial ouabain in this system. Data from Komiyama et al.² with permission.
Figure S7. The two main panels (lower part of the figure) are Figure 2 from Baecher et al., (2013) showing the MS ion chromatogram spectrum for lithiated molecular ions of deuterated ouabain (Ouabain-d3, lower panel) and for a human plasma sample spiked with commercial plant-derived ouabain (upper panel). The baseline in the lower panel is continuous, while the arrows in the upper panel (see Blaustein, 2015) indicate discontinuities where “the signal trace is broken... This inconsistency was introduced by editing the... raw data” (Vogeser & Baecher, 2015). The unedited portion of the latter record, including a peak at 4.99 min (indicated by the arrow with asterisk in the main figure), is shown in Inset “b”. Inset “a” shows the same region from the spectrum of a different human plasma sample, also with a peak at 4.98 min. The inset data are from Figures 1 and 2 in Vogeser & Baecher (2015). Note that the 4.98 min peak in Inset “a” was scaled to 100% whereas that in Inset “b” was reduced by 7.89-fold because the spectrum was scaled to 100% for the ouabain peak. Thus, the 4.98-4.99 min ion peaks in “a” and “b” are approximately the same amplitude and have the same mass/charge (m+Li⁺/z) ratio as ouabain. These ion peaks, edited out of the original data, are likely the recently described polar isomer of EO. These data are reproduced with permission.
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