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ORIGINAL ARTICLE

Coupling biological detection to liquid chromatography: a new tool in drug discovery

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Abstract Procedures to characterize drugs that can be obtained from plant extracts or combinatorial chemistry are tedious, and they consume considerable resources (e.g., animals) and time. Thus, we have looked for a way to streamline this process. We describe here a novel system for the pre-characterization of drugs based on liquid chromatography coupled to biological detection using perifused or perfused organs. This novel system allows the *on-line* detection of pharmacologically active substances in hydrosoluble mixtures from vegetal extracts or combinatorial chemistry libraries. Depending on the volume of drug solution and concentration of the samples, the procedure can work through either medium pressure liquid chromatography or HPLC, and it enables the fingerprints of drugs to be assessed based on their contractile activity on combinations of different isolated tissues. As an example, we show how the system can identify active fractions from an extract of *Stevia rebaudiana* Bertoni, an activity that was later associated with rebaudioside N. Coupling liquid chromatography to biological detection offers a rapid way to focus attention on active products in complex samples, mostly from hydrosoluble species, helping to considerably reduce the time and cost of the pre-characterization of drugs.

Keywords Biological sensors · Drug screening · Natural products · Isolated organs · *Stevia rebaudiana*

Introduction

The usual way to test the biological activity that a given plant may harbor is to start from a crude extract and, should it be of interest, to continue with the tedious, expensive, and time-consuming tasks of fractionating, purifying, and systematically assaying each resulting fraction. This procedure to identify drugs would benefit from methods to restrict such studies to only those fractions where a given biological activity is known to be present.

The effect of substances on isolated tissues has been a particularly powerful way to reveal the pharmacological profile of drugs (Cohen et al. 1994; Vogel 2013). As contraction is one of the main effects of drugs, pharmacologists have employed several, now classic, techniques using isolated tissues to characterize activity on cell receptors (Vane 1964). Although the use of isolated tissues to initially characterize a drug has been largely relegated to a second plane due to the associated costs, the restrictions on animal use and the arrival of new alternatives, these classical preparations are still extremely useful (Vogel 2013).

Liquid chromatography is a popular analytical technique to separate, purify, identify, and quantify substances. Currently, there are columns that allow mobile phases to be used over a wide range of pHs, including physiological solutions that lack organic solvents. Taking advantage of this, we have substituted the typical phosphate buffers normally employed in MPLC (medium pressure liquid chromatography) as a mobile phase

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with a standard physiological balanced saline buffer (Krebs-HEPES). Moreover, instead of standard detectors, the solution eluted from the columns is directed towards classic isolated organ preparations, which allows the biological activity of the species resolved to be monitored continuously.

To test the system, we have used a plant extract from *Stevia rebaudiana* Bertoni (*Asteraceae*), a perennial herb native to the north-eastern region of Paraguay (Duke, 1993). This plant is widely used as a source of sweeteners and for medicinal purposes, especially in regulating glycaemia. Furthermore, the plant's extracts are of value for the treatment of obesity, hypertension, and heartburn and to help maintain uric acid levels low (Chatsudthipong and Muanprasat 2009; Lemus-Mondaca et al. 2012). *Stevia rebaudiana* Bertoni (SR) contains steviosides, diterpene glycosides with a sweet flavor, the main stevioside being 250–300 times sweeter than sucrose (Kinghorn 1987).

In this report, we have set out to establish a system that allowed us to combine the advantages of LC separation of complex mixtures with classical systems to analyze pharmacological activity in isolated organs. We present here data obtained by combining MPLC with organ perfusion (rat kidney), as well as with a perifusion cascade of rat smooth muscle preparations (vas deferens, trachea, aorta, and ileum). In addition, standard analytical detectors can be inserted between the column and the preparation to provide additional information. The fractions where biological activity was detected were collected and further characterized pharmacologically (using a classic organ bath), and their components were identified by mass spectrometry.

Methods

Drugs and chemicals

Unless specified, all drugs were purchased from Sigma-Aldrich (Madrid, Spain). The salts used to prepare the buffers were reagent grade, and pure water was obtained from a Milli-Q Gradient A-10 (Millipore Iberica, Madrid, Spain).

Plant material

Stevia rebaudiana Bertoni was collected in San Rafael (Alto Paraná, Paraguay), and the aerial parts of the plant were air-dried and ground. The powder (950 g) was extracted three times with a mixture of ethanol to water (7:3) using a conventional reflux method for 1 h, and the extract was then filtered and evaporated under reduced pressure. This procedure yielded 19.85 g of extract that was then freeze-dried.

Chromatographic set-up

Although all the experiments reported here were performed using a low-pressure system, a similar set-up was also developed to use on a modified HPLC device (Figs. 1 and 2 of the Supp. Inf.).

As Sephadex G-10, the chosen material for the stationary phase (Hagel 1989), was supplied as a dry powder, before use, it must first be allowed to swell in excess solvent (Krebs-HEPES buffer at room temperature for 24 h). The gel was then packed in an adjustable column in the standard way and when using "rigid" gels like Sephadex G-10 (Nilsson and Nilsson 1974), it is unnecessary to check the operating pressure. A peristaltic pump (ISMATEC, Idex Co, Lake Forest, IL) delivers the Krebs-HEPES solution to the column, previously filtered through a 0.22-μm membrane to remove debris and to enhance sterility (in mM): NaCl (140), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), HEPES (10), glucose (11), pH 7.3 (NaOH). The tissues were superfused with Krebs-HEPES at a rate of 1 mL/min, and maintaining an accurate and controlled flow rate is particularly important when repeating experiments or performing routine preparative work, as well as for the adequate survival of the organs or tissues. As MPLC allows non-degassed solutions to be used, we can aerate the Krebs-HEPES solution reservoir. We used a non-jacketed column (I.D. 2.5 and 30.0 cm length: C5919, Sigma-Aldrich) equipped with a flow adapter (F8767, Sigma-Aldrich) that was filled with the Sephadex G-10 (GE Healthcare) (equivalent to ≈ 40 g of dry Sephadex) to 20 cm (Fig. 1 shows the general scheme of the system).

The fluid emanating from the column is conducted directly to the biological preparations using one six-port injection valve (Diba-Omnifit Sample 1106 injection valve, Sharlab, Barcelona, Spain) with a 1-mL load loop intercalated before the column for sample injection. Another valve, with an injection loop of 800 μL, is placed after the column to calibrate the contractility of the preparations. As an additional control, a spectrophotometer detector set at 254 nm (SPD-6 AV, Shimadzu, Kioto, Japan) recorded the absorbance of the Krebs-HEPES buffer prior to reaching the first organ. The detection system involved either a perfused rat kidney (Fig. 3 SI) or a perifused isolated organ cascade (Fig. 1).

Animals

All organs were obtained from 15 Sprague-Dawley rats (250–300 g) bred at the animal facilities of the University of La Laguna, and their use was authorized by the Ethical Committee (CEIBA) and was in accordance with ARRIVE (McGrath and Lilley 2015) and European Union guidelines (86/609/EEC). Rats were housed in a cage and maintained under a 12/12 h light/dark cycle (lights on 07:00 a.m.) with the ambient humidity at 70–80% and the temperature at

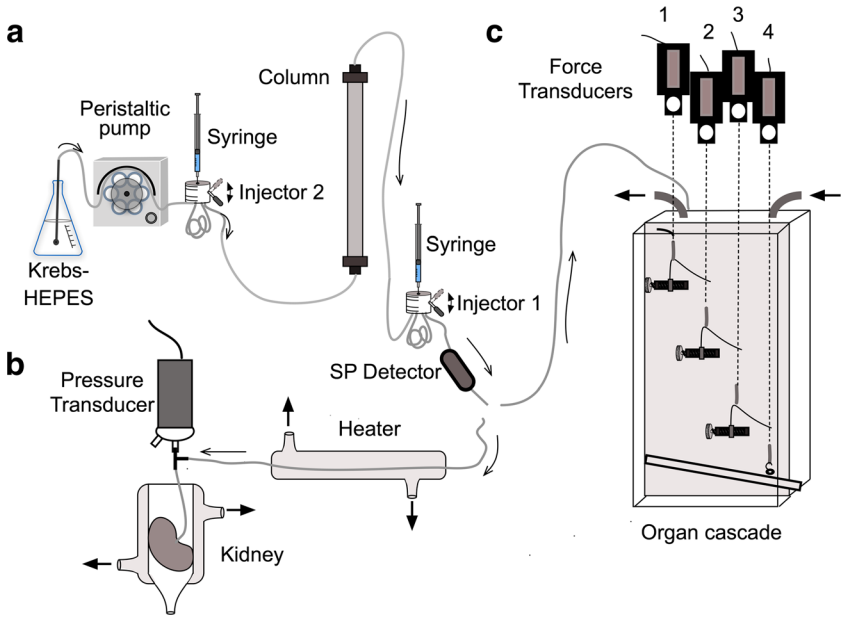

Fig. 1 Liquid chromatography coupled to biological sensors. **A** A Krebs-HEPES solution is pumped through a six-port injection valve (injector 2) using a standard roller pump and then used as the mobile phase in a column. Another injection valve (injector 1) is placed after the column to calibrate the contractile responses. The fluid emanating from the column (gray arrows) is passed through a spectrophotometric detector (SP detector) and then, the fluid can be either conducted to a perfused kidney, the inward pressure of which is continuously monitored **B**, or it can be diverted towards a series of superfused organs (aorta, trachea, vas deferens, ileum: organ cascade) and the tension of each preparation monitored continuously using force transducers **C**. The black arrows indicate the circulation of the warm water used to maintain the preparations at 37 °C

21 ± 2 °C. Food and water were provided ad libitum. For perifused organs, animals were sacrificed by decapitation and their organs were removed rapidly.

Perfused kidneys

Rats were anesthetized with sodium pentobarbitone (50 mg/Kg, i.p.). The abdomen was opened and kidneys identified. Both renal arteries were cannulated with PE10 polyethylene tubing and using a peristaltic pump; they were immediately perfused with Krebs-HEPES buffer that was aerated with O_2 (Borges et al. 1989b). The emanating fluid from renal vein and ureter flowed freely. The kidneys were placed in glass containers warmed with a water jacket to maintain the organ at physiological temperature, and the fluid emanating from the column was also preheated to 37 °C using a water jacket column (Fig. 1). Inflow pressure was continuously monitored using a TRA021 pressure transducer (Harvard-Panlab Instruments, Barcelona, Spain). The arrival of a vasopressor substance to the kidney increased the retrograde pressure.

Superfused chain of organs

We created a system similar to that classically described by John Vane for the serial superfusion of four different organs (Gryglewski and Vane 1972). To minimize the possible downstream artefacts provoked by the release of endogenous substances, the usual order for superfusion in this study was established based on organ weight: aorta, trachea, vas deferens, and ileum. Occasionally, we also used rat portal vein rings or the uterus. The organs were placed in a plastic chamber that had an aluminum surface at the back that was warmed to 37 °C by pumping water from an external bath. The fluid emanating from the column dropped from top to bottom along a silk thread that holds the downstream preparation, bathing the organs, and the correct position to ensure efficient contact with the tissues was adjusted using screw manipulators. Contractions were monitored continuously using isometric force transducers (TRI202P: Panlab SL, Barcelona, Spain), and they were recorded via a four-channel customized bridge amplifier (CANSBRIDGE-4: University of La Laguna, Spain). The data were sampled at 1 Hz and stored on a Mac Mini computer (Apple Inc., Cupertino, CA) using Powerlab 8/35 (AD Instruments, Dunedin, New Zealand) coupled to LabChart 7.3.7 software (AD Instruments). The system was under continuous superfusion and it was left to stabilize for 1 h prior to obtaining measurements. All drugs and plant extracts were dissolved in the Krebs-HEPES buffer, centrifuged, and filtered to eliminate any insoluble particles, thereby protecting the column bed. Only one plant extract was running on each cascade experiment.

Aorta ring chain

The thorax was opened and a 2-cm segment of the rat thoracic aorta was excised carefully cleaned of clots, surrounding fat, and connective tissue, while trying to minimize the mechanical damage. Rings about 2 mm wide were tied together to form a chain of three elements using a U-shaped stainless steel wire (Ø 0.25 mm). A thread was used to attach one end to the holder of the organ chain and the other to the first transducer (see below), and a basal tension of 1 g was applied to the chain of rings (Borges et al. 1989a; Borges et al. 1989b). The tissue was cleaned of clots, surrounding fat and connective tissue, while trying to minimize the mechanical damage.

Tracheal ring chain

The skin of neck was opened by a longitudinal excision and rat trachea segment of ≈ 1 cm was excised, cleaned, and cut into individual rings, which were then tied together to form a chain of four elements, as indicated above for the aorta, with the muscle parts of the tissue aligned to the recording system to maximize the contraction force recordings. The chain was connected to the second transducer of the recording system with a basal tension of 1 g.

Vas deferens

The left testis was exposed by an excision in the scrotum. The vas deferens was exposed and carefully excised avoiding torsion. The prostatic portion of about 1 cm was mounted in the recording system (third transducer) with a basal tension of 0.7 g (Anonymous 1970; Blattner et al. 1978).

Ileum

The abdomen was opened through an incision in the *linea alba*. A 2 cm portion of the last part of the rat ileum was tied, using 4/0 silk thread, to the fourth transducer, leaving the intestinal lumen open to allow the perfusate access to both sides of the tissue. A basal tension of 1 g was applied to this preparation (Anonymous 1970). For further testing, portions of the ileum were mounted in a classic organ bath system within a 4-mL jacketed chamber, and the tissues were aerated with oxygen. Contraction was measured as described above using a customized 16-channel bridge amplifier (CANSBRIDGE-16: University of La Laguna, Spain).

Mass spectrometry

Samples of biological interest were collected and a volume of about 30 mL of saline was dried under vacuum. The resulted extract was resuspended in 500 μL of methanol to be analyzed in negative mode on a mass spectrometer (Micromass LTC Premier XE System, Waters, Mildford, MA, USA). Electrospray ionization (ESI) was assessed by nebulization in pure N_2. The parameters for analysis were as follows: source temperature, 100 °C; desolvation temperature, 150 °C; capillary and cone voltage, 1.9 kV and − 145 V, respectively. Full-scan mass spectra were acquired in the mass *m/z* 100–1500 amu.

Results and discussion

MPLC coupled to biological detectors

For chromatographic separation in an isocratic mode, we had to select a suitable stationary phase that allowed a suitable physiological medium to be used as an eluent that would guarantee the survival of the organs throughout the process. The chromatographic support must also permit the separation of a wide range of compounds, generally low molecular weight entities (< 1000 Da). As such, a standard Krebs-HEPES buffer was selected as the mobile phase while Sephadex G-10 was chosen as the stationary phase. Sephadex G-10 is a gel filtration support formed by cross-linked dextran, and its weak chemical interactions with active molecules make it very suitable for this process, separation occurring essentially on the basis of molecular weight or size (Stokes radius: (Porath and Flodin 1959; Janson 1987)). Of the entire range of G-10 filtration gels, this Sephadex gel is that with the lowest fractionation range (Nilsson and Nilsson 1974).

HPLC-coupled biological detectors

In the "Electronic supplementary material," we show a detailed description of the system when HPLC is used instead of MPLC. HPLC is ideal for very concentrated samples when the use of a small injection volume (< 100 μL) dilutes it beyond the threshold of detection for the organs. In our hands, HPLC has the disadvantage that it requires very concentrated samples or extremely active drugs, such as marine toxins. The HPLC-based system is basically the same as that described here for MPLC, except that a C18 column is used (prontosil, 15 cm, 5 μm: Scharlab) and it is obligatory to degas the mobile phase, making it necessary to use mixed buffers of degassed/oxygenated buffers (Fig. 1 SI). A limitation of the use of reverse phase columns (C2, C8, or C18) is the impossibility of using organic solvents in the mobile phase to elute substances.

Rat kidney as a biosensor

The effect of the mobile phase flow on the discrimination properties of the MPLC column is shown when 0.8 mL of a mixture of the α-sympathomimetic agonists noradrenaline

and phenylephrine is injected (10 μM each, Fig. 3 SI). Even considering that both drugs have a similar molecular weight 169.2 vs. 167.2, the system could discriminate them and especially when the perfusion flow rate decreased.

The superfused organ cascade as a quadruple biosensor

In order to check the responsiveness of the different tissues, we characterized their contractile response when injecting a Krebs-HEPES solution containing 10 μM of well-known drugs like acetylcholine, noradrenaline, serotonin, and adrenaline. Three of these drugs evoked contractile responses in aorta rings (noradrenaline, serotonin, and adrenaline) while both acetylcholine and serotonin did so in tracheal rings (Fig. 2a). Noradrenaline and adrenaline caused the contraction of the vas deferens tissue, and while acetylcholine and serotonin produced contraction in the isolated intestine, noradrenaline and adrenaline relaxed this tissue.

The superfusion system allows the drug to be rapidly washed out of the preparation once it had passed through the organ, and it should be noted that there was a short delay between the substance reaching the first and the last organ (< 5 s). The order of the tissues in the cascade does not seem to be relevant. However, as drugs might cause the release of active substances from some tissues that could in turn affect the tissue downstream in the chain (Gryglewski and Vane 1971), we usually mounted the lighter tissues (like aorta, trachea, or portal vein) higher up in the system, ending with the heaviest tissue (ileum or uterus). No changes in the pH of the buffer were apparently induced by the eluent during the experiment, nor after direct stimulation of the preparation with high K^+ solutions through injector 1. Using this organ chain, we were able to carry out a pharmacological characterization of a natural extract from *Stevia rebaudiana*. In addition to its widespread use as sweetener (Duke 1993; Lemus-Mondaca et al. 2016), some interesting pharmacological effects have been attributed to extracts from the aerial parts of this plant, especially in the intestinal tract (Kinghorn 1987; Chatsudthipong and Muanprasat 2009).

Direct injection of the extract onto the organ chain (10 mg·mL^{-1}) produced a characteristic pharmacological profile involving the contraction of all four organs, although these effects varied when it was injected at a higher concentration (100 mg·mL^{-1}). Although the contraction of the trachea rings was similar at both concentrations, aorta rings experienced a small contraction at this latter concentration followed by a long relaxation. The contraction of the vas deferens was briefer at the higher concentration of the extract and this was not followed by relaxation. Conversely, contraction of the ileum was stronger at this concentration and it was followed by a prolonged relaxation (Fig. 3b). These results suggested that more than one active substance was present in the extract. At the end of the experiment, the responsiveness of preparations was tested by the direct injection of KCl (1 mL, at 70 mM).

To further study the pharmacological effects of the crude extract, 1 mL was injected onto the column at the higher concentration (100 mg·mL^{-1}, see Fig. 1). As the effluent of the column first passed through an absorbance detector set at 254 nm, we always had a temporal reference of the species eluted. Surprisingly, there was no relationship between the time course of absorbance (as obtained from the spectrophotometer detector at 254 nm, and also at 210 and 280 nm). We have also monitored the absorbance at 210 and 280 nm but at these wavelengths, the time course of biological activity was also not consistent. Indeed, the first substance detected by absorbance appeared about 40 min after injection, yet this large absorption peak was not accompanied by any contractile effect (Fig. 3). Conversely, large contractile responses were observed 30 min later, even though no major peak was found

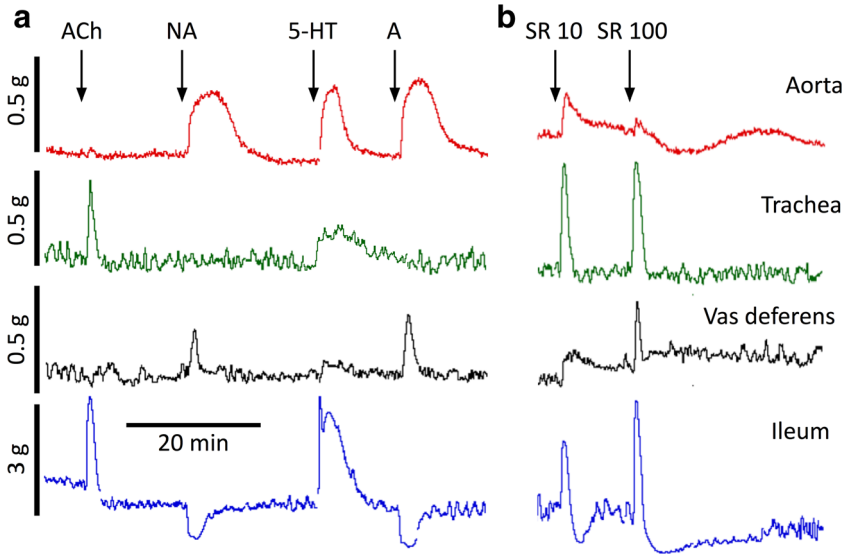

Fig. 2 Drug characterization using four-organ cascade detection. **a**. Direct injection (through injector 2, see Fig. 1) of 1 mL of acetylcholine (ACh), noradrenaline (NA), serotonin (5-HT), and adrenaline (A), all at a concentration of 10 μM. The vertical calibration bars (in grams) are for the force calibration. **b**. Contractile responses to the natural *Stevia rebaudiana* Bertoni (SR) extract at 10 and 100 mg·mL^{-1}, injecting the extract directly through injector 1

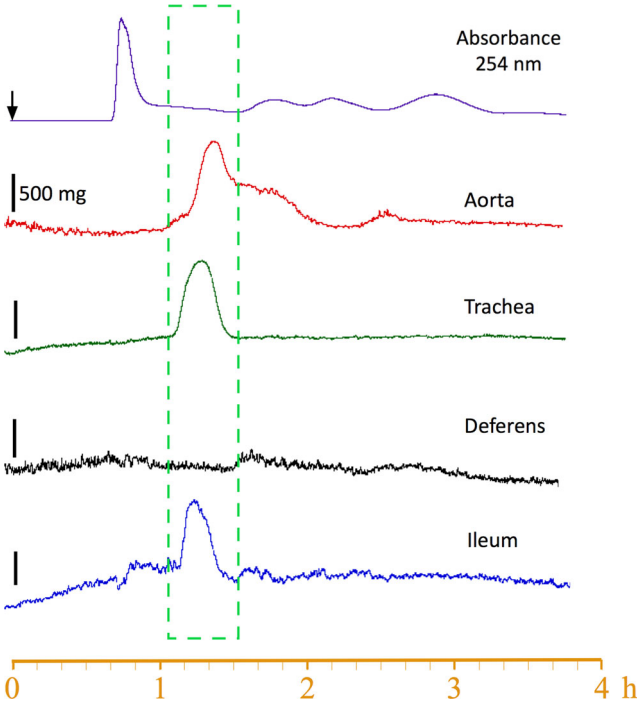

Fig. 3 On-line analysis of *Stevia rebaudiana* Bertoni plant extracts using MPLC coupled to the organ cascade. The aqueous extract (1 mL) was injected into the system and the eluate from the column was directed sequentially to an absorbance detector (set at 254 nm), rat aorta rings, rat tracheal rings change, rat vas deferens, and rat ileum. The eluate from the column that caused the peaks highlighted with a discontinuous box was collected and analyzed by mass spectrometry. The calibration bar corresponds to a 0.5 g tension/force. The figure shows one representative experiment of five. Lower axis corresponds to the time (in hour) from the injection of extract

in the absorbance trace. This should be taken into account when fractions from the separation columns are selected for assays based on a simple chemical analysis of such absorbance. The contractile responses in the four organs differed both in duration and intensity, and the pharmacological profile resembled that observed with serotonin (Fig. 2a).

We used the absorbance recording as a temporal reference in the collection of the eluate from the column 30 min after the first peak, and this fraction was then assayed on isolated rat ileum in a classical tissue organ bath. The extract promoted a biphasic response, with a contraction followed by a long relaxation (Fig. 4a) that suggested the presence of more than one active substance. The process was repeated using only water as the HPLC eluent and significantly, the retention times were not affected by this change in the eluent. The fraction with the same retention time was now subjected to a new analysis in an organ bath to verify that it matched the biological activity. After a quick separation, the fraction was desiccated and the dry product was resuspended in 1 mL of methanol and centrifuged.

As only the soluble fraction exhibited activity in the ileum, this fraction was analyzed by mass spectrometry in negative mode and an ion at *m/z* 1273.5336 $[M\text{-}H]^-$ (Calculated for $C_{56}H_{89}O_{32}$ 1273.5337) was detected (Fig. 4b and 4 SI). This ion matches the previously reported steviol glycoside rebaudioside N (Fig. 4c: (Ohta et al., 2010, Ceunen and Geuns 2013; Prakash et al. 2014)). To the best of our knowledge, the activity of this rebaudioside has not previously been tested on smooth muscle preparation (Madan et al. 2010). Figure 4 shows several compounds were detected in the fraction collected (as indicated by the box with a discontinuous line in Fig. 3). As rebaudioside N is not commercially available, we cannot rule out the biological activity of other compounds in the extract. However, we think that rebaudioside N is the main candidate as stevioside and rebaudioside A are thought to be vasodilators (Chatsudthipong and Muanprasat 2009) or to have no effect on blood pressure (Maki et al. 2008), whereas the product identified in the active fraction is a potent vasoconstrictor.

In this article, we describe a novel system for the pharmacological characterization of products derived from natural plant extracts. This new method has several advantages over the classic way of testing natural products, avoiding tedious, expensive, and time- and animal-consuming procedures. Prior to that, we use to start analyzing the effects of a given whole plant extract. Usually, the presence of substances with opposite actions masked the occurrence of a drug. Then, we had to start the fractioning procedure, than mean producing large amounts of starting material frequently from not abundant plants. Sometimes to produce enough extract, it became necessary culturing plants in danger of extinction. Now, instead of fractionating, purifying, and systematically assaying each resulting fraction, this procedure allows attention to focus only on those fractions in which biological activity is found. This invention has largely reduced the animal (15 vs. 40 rats) consumption and speeds up the study (4 weeks vs. 6 months) to carry out the detection, characterization, and identification of an active substance. That could be done because our system excluded from the study many molecules lacking of our pharmacological interest: targeting drugs for contractility of smooth muscle.

The system has some limitations due to the capacity of the columns to separate compounds. Although mobile phases with organic solvents (acetonitrile, methanol) cannot be used directly for biological preparations, the continuous improvements in separation techniques will help enhance the biological detection of non-polar elements. This latter limitation restricts the capacity for separation to molecular size, although the use of the simple criterion of the Stokes radius allows us to vary and select different supports, G-10, G-15, or G-25, expanding the discriminative features of the columns. No biological activity was found neither in the "front" of the chromatogram (high molecular weight) nor along the 60 min after the last absorbance peak, at 254 nm, was detected (Fig. 3). Although it is possible that given species can be retained in the

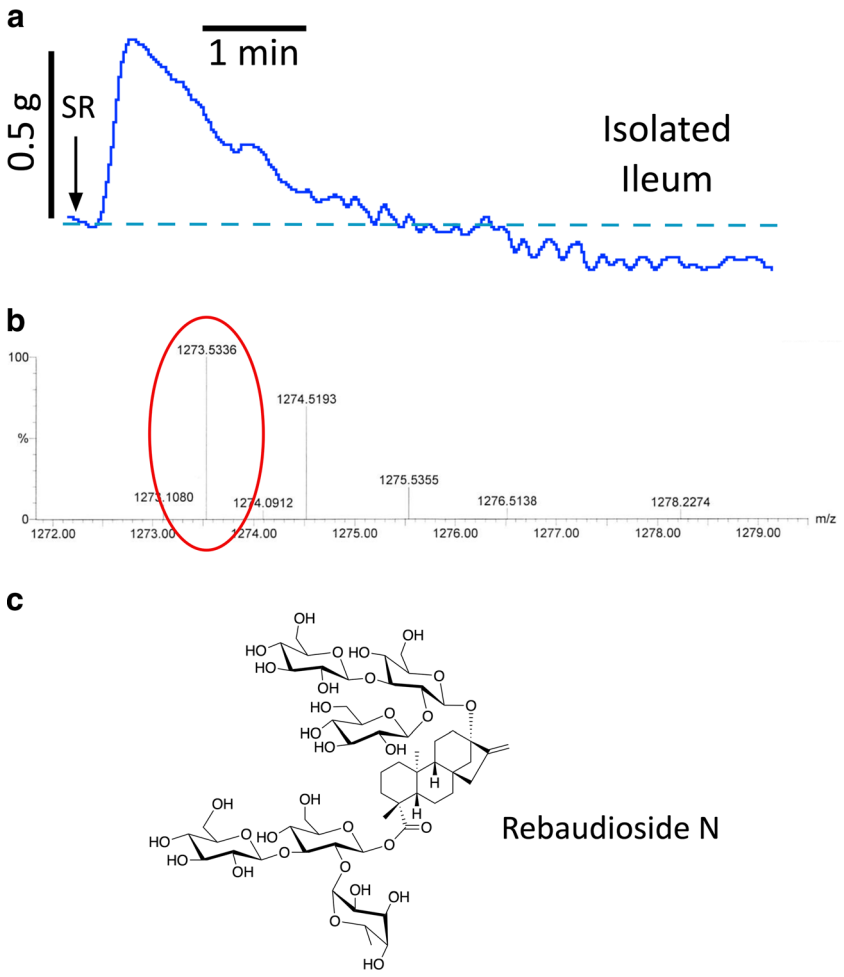

Fig. 4 Characterization of the active fraction. The eluate from the column where the pharmacological activity was found was collected (10 mL), concentrated, and resuspended in water. **a**. An aliquot (60 μL) of this solution (SR) was added to an isolated rat ileum in a 4-mL classic organ bath chamber at the time indicated by the arrow. The traces are representative from four different experiments. **b**. Electrospray high resolution mass spectrometry spectrum of the methanol-soluble fraction. **c**. Chemical structure of rebaudioside N

Sephadex bed, it is unlikely that they are quantitatively important provided they were not escape after washing out the column. As organs cannot be perfused with extracts at different salt concentrations or pH, this also rules out the possibility of using of resin-exchange stationary phase. Nevertheless, we hope that current improvements in separation technologies will overcome this problem.

As the samples to be analyzed must be completely soluble in saline buffer at the moment of injection, this rules out the direct injection of organic extracts. The solubility of non-polar extracts is frequently a problem for pharmacological studies on isolated organs. The need to use solvents like dimethyl sulfoxide (DMSO) limits the study of many extracts due to their toxicity. Nevertheless, perfused organs can be safely exposed to solubilized samples on the condition that at the moment of reaching the organs, the proportion of solvents like methanol, ethanol, or DMSO is below 0.1%. This means that most vegetable will be suitable for such on-line analysis. Our system was particularly useful for hydrosoluble extracts. Our Sephadex column retains little of this Stevia extract and indeed, when the columns were cleaned with 10% methanol, there was no appreciable retention of polar species.

Although the period of the year is crucial for plant collection, it seems not important for a perennial plant like *Stevia rebaudiana* Bertoni (Duke 1993). The steps followed for obtaining the extracts are standard procedures using organic solvents (Vazdekis et al. 2009). As in all plant extraction procedure, it would exclude insoluble compounds. Nevertheless, the system allows to first describing pharmacological activity of rebaudioside N (Fig. 4 B&C).

We currently also use other tissues from rats such as uterus (for assay drugs acting on uterine motility), gastric fundus strips (for prokinetic drugs), or portal vein (for preload acting agents) can be used at the organ chain instead of the already described organs. Also, local slaughterhouses can provide a number of tissues from rabbits, chicken, pigs, or cow that will reduce the experimental animal consumption.

The direct coupling of chromatography to biological detection has proved to be very useful to facilitate the pharmacological characterization of active compounds in mixtures, as evident here with rebaudioside N. Although no experimental approach can describe the full pharmacological characteristics of a drug, this system can characterize a range of drug

activities, both the acute activity and the toxicity of the eluted substances.

Contractile activity involves a wide range of mechanisms implicated in the stimulus-contraction coupling, including those driven by receptors, ion channels, second messengers, and contractile proteins. These can all be assessed in four, or more, well-characterized preparations. Contractile activity involves a wide range of mechanisms implicated in the stimuli-contraction coupling as it includes receptors, channels, second messengers, and contractile proteins in four, or more, well-characterized preparations.

Thus, this approach opens new and exciting possibilities in the field of drug research.

Author contributions MACB and RGB: isolated the plant extracts and performed the experiments in the isolated organ bath. MC, JGHJ, and RGB: performed the experiments on the isolated organ cascade. RGB and JJF: performed the analyses with the gel filtration system and mass spectrometry. MSM: performed the experiments on the perfused kidneys. FGD: isolated the plant extracts. RB: conceived the idea and write the paper.Funding informationThis work was supported by the Spanish Ministry of Economía y Competitividad (MINECO) grants (BFU2013-45253-P) to RB and (CTQ2014-55888-C03-01/R) to JJF. MC was recipient of a travel stipend from the Government of Paraguay (Consejo Nacional de Ciencia y Tecnología, CONACYT). RGB is recipient of a FPU fellowship form MINECO.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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