Coupling biological detection to liquid chromatography

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Running title: Chromatography with biological detection

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ABSTRACT

Background and Purpose: Procedures for drug characterization obtained from plant extracts or combinatorial chemistry are tedious, time- and animal consuming.

Experimental Approach: We describe here a novel system for pre-characterization of drugs based in liquid chromatography coupled with biological detection based on perifused or perfused organs.

Key Results: This novel system allows the *on-line* detection of pharmacologically active substances present in hydrosoluble mixtures from vegetal extracts or combinatorial chemistry. Depending on the volume and concentration of samples, the procedure can work on either LPLC or HPLC and allows obtaining the fingerprints of drug activity based on the contractile activity of a combination of different isolated tissues. As an example we show here the capabilities of the system for identifying active fractions from a vegetal extract of *Stevia rebaudiana Bertoni* that they was latterly identified as rebaudioside N.

Conclusions and Implications: Coupling liquid chromatography with biological detection offers a rapid way to centre the research of active products present in complex samples. This will largely reduce the time and cost of pre-characterization of drugs.

INTRODUCTION

The usual way for testing the biological activity of a given plant is starting from a crude extract and, if it results were of interest, continuing with the tedious, expensive and time-consuming tasks of fractioning, purification and systematic assay of each resulting fraction. This procedure is currently the same for complex mixtures resulting from modern synthetic methods such as combinatorial chemistry. The procedure for drug characterization will be beneficed whether one can restrict the study to just only those fractions where biological activity was present.

The effects of substances on the contractile activity of isolated tissues have been one of the most powerful classical tools for unveiling the pharmacological profile of drugs. As contraction is one of the main effector of drugs, pharmacologists have implemented several, now classic, isolated tissue techniques to characterize many drug action on tissue receptors. Although currently the use of isolated tissues as first tool for drug characterization has been largely reduced due to the cost, animal use restrictions and the arrival of new alternatives (such as cell culture, immobilized targets and *in-silicon* simulations) these classical preparations are still nowadays extremely useful.

Liquid chromatography is probably the most popular analytical technique for separation, purification, identification and quantification of substances. The arrival of HLPC, LPLC and FPLC with reverse-phase columns made these systems compact and highly versatile when coupled with analytical detectors (fluorescence, absorbance, electrochemical, mass-spectrometry…) capable to identify the species which, based in their physic-chemical characteristics, sequentially emanate from the columns. Currently, there are columns available that allow the use of mobile phases with a wide range of pH, which would also include physiological solutions (Krebs', Tyrode's, Locke's…) lacking in organic solvents. Taking this advantage, we have substituted the typical phosphate buffers normally employed as mobile phase by a standard physiological balanced saline buffer (Krebs-HEPES). Instead of standard detectors, the solution escaping from columns is directed towards classical organ isolated preparations and the biological activity of the resolved species continuously monitored.

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 for sweetener compounds and for medicinal purposes, especially for the regulation of glycaemia. Also, plants extracts have reveal of

value for the treatment of obesity, hypertension, heartburn, and to help to keep low uric acid levels (Chatsudthipong & Muanprasat, 2009; Lemus-Mondaca, Vega-Galvez, Zura-Bravo & Kong, 2012). It contains steviosides, diterpenes glycosides, which are sweet components, being the main the stevioside, 250-300 times sweeter than sucrose (Kinghorn, 1987).

In this report we present data using LPLC combined with perfused organs (rat kidney) as well as a perifusion cascade of rat smooth muscle preparations (vas deferens, trachea, aorta and ileum). In addition, standard analytical detectors can also be inserted between the column and the preparation to provide extra information. The fractions where biological activity was detected were collected and further characterized pharmacologically (classical organ bath) and identified by mass spectrometry analysis.

METHODS

Drugs and chemicals. Unless specified, all drugs were purchased from Sigma-Aldrich (Madrid, Spain). Salts used for preparing 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. The aerial parts were air dried and grounded. The powder (950 g) was extracted three times with a mixture of ethanol:water (7:3) by a conventional reflux method for 1 h, filtered and evaporated under reduced pressure yielding 19.85 g of extract. The concentrated extract was freeze-dried.

Chromatographic set-up. Although all the experiments related in this paper have been done using a low-pressure system, a similar set-up was also developed for using on a modified HPLC device (Figures 1 & 2 of the SI).

As Sephadex G-10, the chosen material for stationary phase (Hagel, 1989), was supplied as a dry powder; this must be allowed to swell in excess solvent (Krebs-HEPES buffer at room temperature for 24 h) before use. The gel was packed, in the standard way, in an adjustable column. When "rigid" gels such as Sephadex G-10 (Nilsson & Nilsson, 1974) are used, precaution such the checking of the operating pressure is unnecessary.

Figure 1 shows the general scheme of the system. A peristaltic pump (ISMATEC, Idex Co, Lake Forest, IL) delivers Krebs-HEPES solution (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 buffer was previously filtered through a 0.22 µm membrane to remove debris and to reduce the bacterial grown. Tissues superfused with Krebs-HEPES at 1 mL/min. Accurate and control of flow rate is particularly important when repeating experiments or performing routine preparative work and the correct survival of the organs or tissues. As LPLC allowed the use of non-degassing solutions we can aerate the Krebs-HEPES solution reservoir. We have used a non-jacked column I.D. 2.5 cm and 30.0 cm length (C5919, Sigma-Aldrich) equipped with a flow adapter (F8767, Sigma-Aldrich), filled with Sephadex G-10 (GE Healtcare) to 20 cm length.

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

Animals. All organs were obtained from Sprague-Dawley rats breaded in the animal facilities of the University of La Laguna and its use was authorized by the Ethical Committee and was in accordance with the guidelines of the European Union (86/609/EEC). Animals were killed by decapitation and organs rapidly removed.

Perfused kidneys. Both renal arteries were cannulated with a polyethylene tubing and immediately perfused using a peristaltic pump with Krebs-HEPES buffer aerated with O_2 (Borges, von Grafenstein & Knight, 1989). Kidneys were place in a jacket glass container to maintain the organ at physiological temperature. Also, the fluid emanating from the column was preheated at 37 ºC by a water jacket column (Figure 1).

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 & Vane, 1972) (Figure 1C). Spanish patent # ES2372832 A1 (27.01.2012). To minimize the

possible artefact caused downstream by the release of endogenous substances, the usual order for the superfusion for this study was selected by organ weigh: aorta, trachea, vas deferens and ileum. Occasionally, we also used rat portal veins rings or uterus. The organs were placed in a plastic cupboard that had a back aluminium surface warmed at 37ºC by pumping water from an external bath. The emanating fluid dropped from top to bottom bathing the organs. The correct position, to assure an efficient contact with the tissues, was assessed by screw manipulators. Contractions were continuously monitored by isometric force transducers (TRI202P, Panlab SL, Barcelona, Spain) and recorded using a 4-channels customized bridge amplifier (CANSBRIDGE-4, University of La Laguna, Spain). Data were sampled at 1 Hz and stored in a Mac-Mini computer (Apple Inc, Cupertino, CA) using a Powerlab 8/35 (AD Instruments, Dunedin; New Zealand) coupled to a LabChart 7.3.7 software (AD Instruments). The system under continuous superfusion was leaved to stabilize for one hour. All drugs and plant extracts were dissolved in the Krebs-HEPES, centrifuged and filtered to eliminate insoluble particles to protect the bed of the column.

Aorta rings chain. A 2 cm segment of rat thoracic aorta was excised and cut to obtain rings of about 2 mm wide. The rings were then tied together using a U-shape stainless steel wire (Ø 0.25 mm) to form a chain of 3 elements. A thread is used to attach one end to the holder of the chain of organs and the other to the first transducer (see below). The chain of rings received a basal tension of 1 g (Borges, Carter, von Grafenstein, Halliday & Knight, 1989; Borges, von Grafenstein & Knight, 1989). Tissues were cleaned of clots, surrounding fat and connective tissue trying to minimize the mechanical damage.

Tracheal rings chain. Rat trachea was excised, cleaned and cut into individual rings. The rings were then tied as above to form a chain of 4 elements leaving the muscular parts aligned to the recording system. The chain was connected to the second transducer of the recording system and with a basal tension of 1 g.

Vas deferens. The tissue is excised and the prostatic portion of about 1 cm was mounted in the recording system (third transducer) with a tension of 0.7 g (Anonymous, 1970; Blattner, Classen, Dehnert & Döring, 1978).

Ileum. A 2 cm portion of the last portion of the rat ileum was tied to the fourth transducer leaving the lumen open for allowing the perfusate accessing both sides of the tissue. A tension of 1 g was applied to the preparation (Anonymous, 1970). For further

testing, portions of ileum were mounted in a classical organ bath system with a 4 mL jacketed chamber; the tissues were aerated with oxygen. Contraction was measured as described above using a customized 16-channels bridge amplifier (CANSBRIDGE-16, University of La Laguna, Spain).
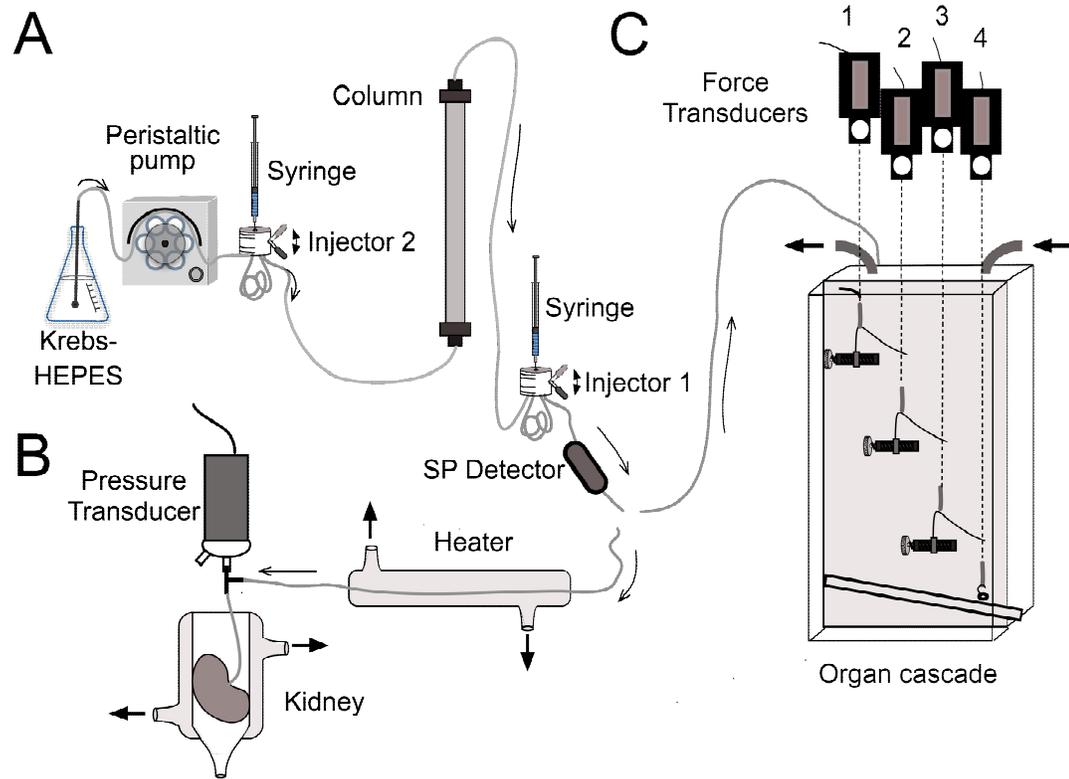
Figure 1. Liquid chromatography coupled with biological sensors. A, Krebs-HEPES solution is pumped using a standard roller pump. The buffer is passed through a 6-port injection valve (Injector 2) and then used as mobile phase in a column. Another injection valve (Injector 1) is placed after the column for the calibration of contractile responses. The emanating fluid (grey arrows) is passed through a spectrophotometric detector (SP Detector), then fluid can be either conducted to a perfused kidney and the inward pressure continuously monitored **(B),** or diverted towards a series of superfused organs - aorta - trachea - vas deferens – ileum - (Organ cascade) and the tension from each preparation continuously monitored using force transducers **(C).** Black arrows indicate the circulation of warm water used for thermostatizing the preparations at 37ºC.

RESULTS AND DISCUSSION

For chromatographic separation, in an isocratic mode, we had to select a suitable stationary phase that allowed the used as eluent a suitable physiological medium that guarantees the survival of the organs throughout the process. Chromatographic support must also allow the separation of a wide range of drugs, generally low molecular weight entities (<1,000). In the present work, standard

Krebs-HEPES buffer was selected as the mobile phase whereas Sephadex G-10 was chosen as stationary phase.

Sephadex G-10 is a gel filtration support formed by cross-linked dextran, which is very suitable for this process due to its low chemical interaction with the molecules since a separation occurs basically due to its molecular weight or size (Stokes radius) (Janson, 1987; Porath & Flodin, 1959). Of the entire range of G-10 filtration gels is the one with the lowest fractionation range (Nilsson & Nilsson, 1974).

HPLC-coupled biological detectors. In the Supporting Information we show a detailed description of the system when HPLC is used instead of LPLC. HPLC results ideal for highly concentrated samples when the dilution caused by the small volume of injection (<100 µL) is above the detection threshold of the organs. In our hands, HPLC has the disadvantage of requiring highly concentrated samples or extremely active drugs such as marine toxins. HPLC-based system is basically the same described here, except from the column used C18 column (Prontosil, 15 cm, 5 µm, Sharlab) and the compulsory use of degassing mobile phase, which obliged to use mixed buffers of degassing/oxygenated buffers (Figure 1 SI).

Rat kidney as a biosensor. Figure 3 SI shows the effect of mobile phase flow on the discrimination properties of the LPLC column. We injected 0.8 mL mixture of the α-sympaticomimetic agonists noradrenaline and phenylephrine (10 µM each). Even considering that both drugs have with similar molecular weights 169.2 *vs.* 167.2 the system was able to discriminate them specially when the perfusion flow decreased.

The superfused organ cascade as a quadruple biosensor. In order to check the responsiveness of the different tissues we characterized the contractile responsiveness by injecting Krebs-HEPES solution containing 10 µM of well-known drugs like acetylcholine (ACh), noradrenaline (NA), serotonin (5-HT) and adrenaline (A). Figure 2A shows the typical fingerprint of these drugs as NA, 5-HT, and A evoked contractile responses in aorta rings whereas Ach and 5-HT did it in tracheal rings. Noradrenaline and A caused the contraction on vas deferens. In the isolated intestine, ACh and 5-HT produced contraction, whereas NA and A relaxed the tissue.

The superfusion system allows the rapid washout of the preparation once the drug has passed through the organ. There was a short delay (<5 s) since a substance reached

the first and the last organ. As drugs might cause the release of active substances from some tissues (Gryglewski & Vane, 1971). As it is likely that some drugs can cause the release of active mediators from tissues situated downstream, we usually mounted the lighter tissue in the first place (i.e. aorta) and the heavier (i.e. ileum) in the last position.

Using this organ cascade we carried out a pharmacological characterization of a natural extract of *Stevia rebaudiana*. In addition of its wide use as sweeter agent (Duke, 1993; Lemus-Mondaca, Ah-Hen, Vega-Galvez, Honores & Moraga, 2016), the extracts from the aerial parts of the plant have shown to possess some interesting pharmacological effects, especially in the intestinal tract (Chatsudthipong & Muanprasat, 2009; Kinghorn, 1987).
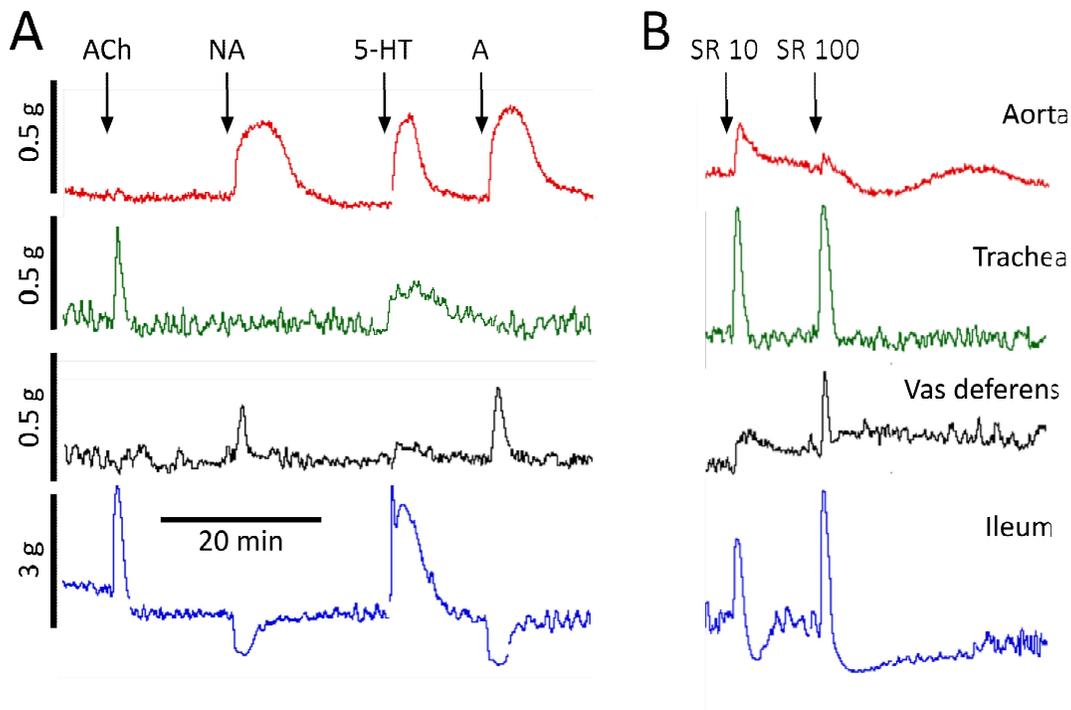

Figure 2. Drug characterization using the four-organ cascade detection. A, Direct injection (through Injector 2, see figure 1) of 1 mL of acetylcholine (ACh), noradrenaline (NA), serotonin (5-HT), adenosine triphosphate (ATP), histamine (His) and adrenaline (A) all at 10 µM. Vertical calibrations (in grams) bars are for force calibration. **B,** Contractile responses to the natural extract of *Stevia rebaudiana Bertoni* (SR) 10 and 100 mg·mL^{-1}, drug was directly injected through injector 1. Upper trace is the absorbance recording obtained from the spectrophotometric detector set at 254 nm. Vertical bar indicates the arbitrary units of absorbance. At the end of the experiment the responsiveness of the system was calibrated by the injection of 1 mL of Krebs-HEPES containing KCl 70 mM (accompanied by the isosmolar reduction of NaCl, not shown).

The direct injection of the extract to the organ chain produced a characteristic pharmacological profile consisting in contraction in all four organs when tested at 10 mg·mL^{-1}. However, these effects varied the moment that 100 mg·mL^{-1} was injected. At this concentration, contraction in trachea rings was similar to that observed at 10

mg·mL^{-1}. Conversely, aorta rings experienced a small contraction followed by a long relaxation. In the vas deferens, the contraction was briefer and was not followed by relaxation. On the ileum, contraction was higher and was followed by a prolonged relaxation (Figure 3B). These results indicated that probably more than one active substance were present in the extract.

At the end of the experiment, the responsiveness of preparations was tested by the direct injection of 1 mL of 70 mM KCl.

For the further investigation of the pharmacological effects of the crude extracts we injected 1 mL (at concentration 100 mg·mL^{-1}) in the column as described in figure 1. As the effluent of the column firstly passed through an absorbance detector we had always a temporal reference of the escaped species. Surprisingly, there were no relation between the time course of absorbance (given by the spectrophotometer detector) and the pharmacological activity. Figure 3 shows that the first substance detected by absorbance appeared at about 40 min after injection. However, this large absorption peak was not accompanied by any contractile effect. Conversely, large contractile responses were observed 30 min later even though no large signal was found in the absorbance trace. This should be taking into account when fractions from separation columns are selected for assay based on a simple chemical analysis such absorbance.
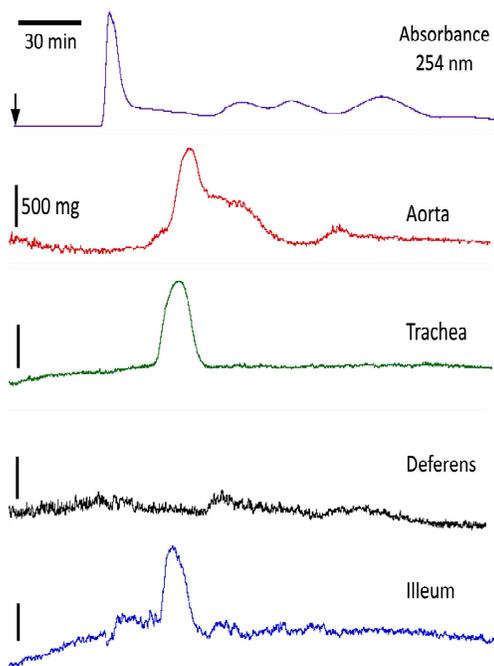

Figure 3. On-line analysis of plant extract (*Stevia rebaudiana Bertoni*) using LPLC coupled with the organ cascade. One mL of the aqueous extract was injected in the system and the effluent from the column directed sequentially to an absorbance detector (set at 254 nm), rat aorta rings, rat tracheal rings change, rat was deferens and rat ileum. The elution from column, which caused the peaks highlighted with discontinuous box was collected and analyzed by MS. Calibration bar corresponds to a 0.5 g tension/force.

The contractile responses in the four organs were different both in duration and in extend. This pharmacological profile resembles what have been observed with 5-HT (Figure 2A).

We used the absorbance recording as temporal reference, for collecting the effluent of the column 30 min after the first peak. This fraction was then used for assay

on isolated rat ileum on a classical tissue organ bath. Figure 4A shows that extract promoted a bi-phasic response with a contraction followed by a long relaxation, thus suggesting the presence of more than one active substance. The process was repeated using only water for HPLC as eluent. 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 organ bath for verifying that it matched identical biological activity. For a quick separation, the fraction was desiccated and the dry product re-suspended in 1 mL of methanol and centrifuged. As only the soluble fraction exhibited activity in ileum. This fraction was analysed by mass spectrometric analysis in negative mode and an ion at m/z 1273.5336 [M-H]$^-$ (Calculated for $C_{56}H_{89}O_{32}$ 1273.5337) was observed (figure 4B and 4 SI). This ion fit whit the previously reported for a steviol glycoside rebaudioside N (Ceunen & Geuns, 2013; Ohta et al., 2010; Prakash et al., 2014) (Figure 4C).

To the best of our knowledge, the activity of this rebaudioside has not been tested on smooth muscle preparation (Madan et al., 2010).
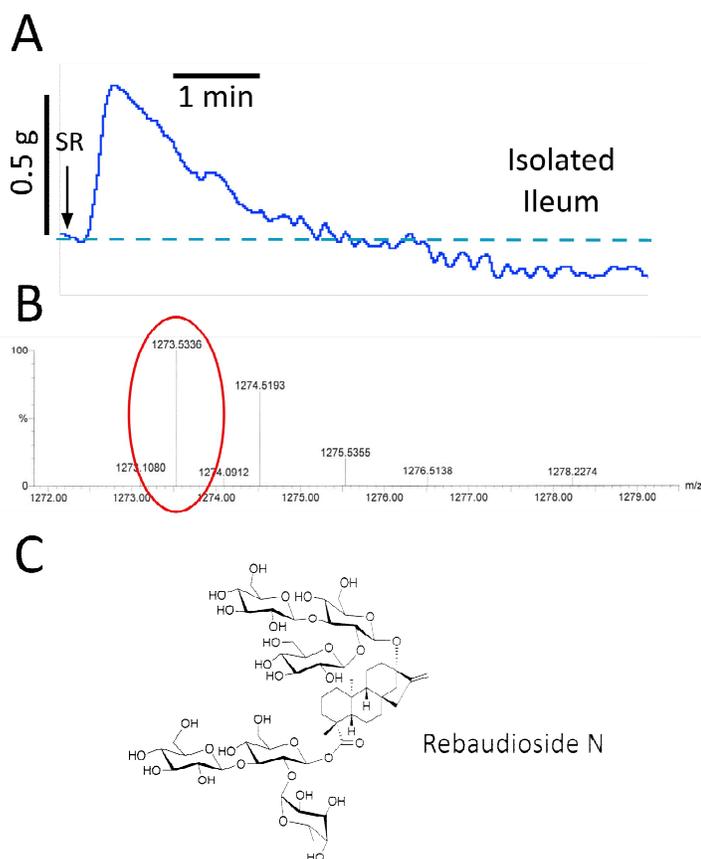
Figure 4. Characterization of the active fraction. 10 mL of the effluent from the column, where pharmacological activity was found, was collected concentrated and re-suspended in water. A, 60 µL of this solution (SR) were added to an isolated rat ileum placed in a 4 mL chamber of a classical organ bath at the time indicated by the arrow. **B,** ESI-HRMS spectrum of the methanol-soluble fraction **C,** Chemical structure for rebaudioside N.

In this paper we have described a novel system for the pharmacological characterization of products resulting from natural plant extracts. This procedure will also be highly useful for the study of substances generated

by combinatorial chemistry. This new method provides several advantages over the classical way of testing natural products avoiding the tedious, expensive, time- and animal consuming procedures. Instead of fractioning, purification and systematic assay of each resulting fraction, this allows focusing the attention only in those fractions where biological activity is found.

The system has yet some limitations due to the separation capability of columns and the impossibility of use standard mobile phase, which contains toxic organic solvent (acetonitrile, methanol), this currently restrains the separation capability almost solely based on molecular size. However, the use of the simple criteria of Stokes radius allows us to vary and select in different supports G-10, G-15 or G-25, which will expand the discriminative features of columns. As organs cannot be perfused with different salt concentrations or pH this also exclude the use of resin-exchanger stationary phase. Nevertheless, we hope that current improvements in separation technologies will overcome this problem.

As, samples must be completely soluble in saline buffer at the moment of injection, this exclude direct injection of organic extracts. Nevertheless, perfused organs can safely accept solubilized samples on condition that solvents like methanol, ethanol or dimethyl sulfoxide (DMSO), at the moment of reaching the organs, were below 0.1%. This means that most vegetal extracts or mixtures produced by combinatorial chemist will be suitable for this on-line analysis.

The use of chromatography directly coupled to biological detection has demonstrated highly useful for shortening the pharmacological characterization of active compounds present in mixtures as has occurred in our hands with rebaudioside N. This concept can be used also for single cell recording coupled to HPLC to detect activity on channels and receptors by calcium indicators or electrophysiological recordings. These possibilities opens new and exciting field for drug research.

AUTHORS CONTRIBUTION

MACB and RGB: isolated the plant extracts and performed experiments in isolated organ bath. MC, JGHJ and RGB: performed the experiments on isolated organ cascade. RGB and JJF: performed the gel filtration system and mass spectrometry analyses.

MSM: performed the experiments on perfused kidneys. FGD: isolated the plant extracts. RB: conceived the idea and write the paper.

CONFLICT OF INTEREST STATEMENT

Authors declare not conflict of interest.

SUPPORTING INFORMATION

Four figures (SI), legends and explanatory text.

REFERENCES

Anonymous (1970) *Pharmacological experiments on isolated preparations*. 2nd edn. E&S Livingstone: Edinburgh.

Blattner R, Classen H, Dehnert H, & Döring H (1978) *Experiments on isolated smooth muscle preparations*. Hugo Sachs Elektronik KG: Hugstetten.

Borges R, Carter DV, von Grafenstein H, Halliday J, & Knight DE (1989). Ionic requirements of the endothelin response in aorta and portal vein. Circ Res 65**:** 265-271.

Borges R, von Grafenstein H, & Knight DE (1989). Tissue selectivity of endothelin. Eur J Pharmacol 165**:** 223-230.

Ceunen S, & Geuns JM (2013). Steviol glycosides: chemical diversity, metabolism, and function. J Nat Prod 76**:** 1201-1228.

Chatsudthipong V, & Muanprasat C (2009). Stevioside and related compounds: therapeutic benefits beyond sweetness. Pharmacol Ther 121**:** 41-54.

Duke JH (1993). Stevia rebaudiana. In CRC Handbook of Alternative Cash Crops. pp 422-424.

Gryglewski R, & Vane JR (1971). Rabbit-aorta contracting substance (RCS) may be a prostaglandin precursor. Br J Pharmacol 43**:** 420P-421P.

Gryglewski R, & Vane JR (1972). The release of prostaglandins and rabbit aorta contracting substance (RCS) from rabbit spleen and its antagonism by anti-inflammatory drugs. Br J Pharmacol 45**:** 37-47.

Hagel L (1989). Gel filtration. In Protein Purification Principles, high resolution methods, and applications. ed L.Rydén J.-C.J. VCH Publishers Inc. New York., pp 63-106.

Janson J (1987). On the history of the development of Sephadex. Chromatographia 23**:** 361-369.

Kinghorn AD (1987). Biologically active compounds from plants with reputed medicinal and sweetening properties. J Nat Prod 50**:** 1009-1024.

Lemus-Mondaca R, Ah-Hen K, Vega-Galvez A, Honores C, & Moraga NO (2016). Stevia rebaudiana Leaves: Effect of Drying Process Temperature on Bioactive Components, Antioxidant Capacity and Natural Sweeteners. Plant Foods Hum Nutr 71**:** 49-56.

Lemus-Mondaca R, Vega-Galvez A, Zura-Bravo L, & Kong AH (2012). Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. Food Chem 132**:** 1121-1132.

Madan S, Ahmad S, Singh GN, Kohli K, Kumar Y, Singh R*, et al.* (2010). Stevia Regaudiana (Bert.) Bertoni - A Review. Ind J Nat Prod Resourc 1**:** 267-286.

Nilsson G, & Nilsson K (1974). Molecular-weight distribution determination of clinical dextran by gel permeation chromatography. J Chromatogr 101**:** 137-153.

Ohta M, Sasa S, Inoue A, Tamai T, Fujita S, Morita K*, et al.* (2010). Characterization of novel steviol glycosides from leaves of Stevia rebaudiana Morita. J Appl Glycosci 57**:** 199-209.

Porath J, & Flodin P (1959). Gel filtration: a method for desalting and group separation. Nature 183**:** 1657-1659.

Prakash I, Bunders C, Devkota KP, Charan RD, Ramirez C, Priedemann C*, et al.* (2014). Isolation and characterization of a novel rebaudioside M isomer from a bioconversion reaction of rebaudioside A and NMR comparison studies of rebaudioside M isolated from Stevia rebaudiana Bertoni and Stevia rebaudiana Morita. Biomolecules 4**:** 374-389.