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Coupling Biological Detection to Liquid Chromatography as a Solid Tool for the Separation, Purification and Online Biological Characterization of Chemical Compounds Present in Natural or Synthetic Mixtures †

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Abstract: Current methodologies for separating, isolating, purifying, and identifying biological properties of compounds present in mixtures are very long and complicated. This task is further complicated if chemical compounds with similar physicochemical properties coexist during the separation, fractionation, isolation, and purification conditions. An effective and widely used tool in these procedures is chromatography. In previous years, our research group has achieved the pre-characterization of bioactive molecules by developing and optimizing a liquid chromatography technique directly coupled to studies of activity determination in isolated living organs. We tested the usefulness of this system by determining the activity of rebaudioside N, a natural product derived from an extract of the *Stevia rebaudiana* Bertoni plant.

Keywords: drug discovery; natural products; synthetic mixtures; isolated organs; *Stevia rebaudiana*

1. Introduction

The direct interaction of molecules, extracts and medicines with tissues or living organisms it is essential and unavoidable knowledge in the actual commercial therapy. Studies achieve during the preclinical and clinical stages of drug development and that are very useful to reveal the pharmacological profile of drugs.

In most cases, pure chemical compounds are obtained by artificial synthesis, which results in mixtures of compounds, or are isolated from sources of natural origin. Both methods involve subsequent isolation and purification processes. But the actual process for separating, isolating, purifying and identifying biological properties of compounds present in mixtures are very complex and require a lot of work time.

In this paper, we describe the establishment of a system that allows to combine the advantages of liquid chromatography (LC) separation of complex mixtures with classical systems to analyze pharmacological activity in isolated organs. The development and optimization of this system were useful to isolate, purify and identify the biological activity of the natural product rebaudioside N. A minor compound produced by the plant species *Stevia rebaudiana* Bertoni [1].

The proven effectiveness of this methodology allows describe uses and perspectives in bioorganic chemistry of coupling LC (for examples, MPLC, medium pressure liquid chromatography, or HPLC, high performance liquid chromatography) to studies of living tissues or organs in the pre-characterization processes of new bioactive compounds present in mixtures.

2. Materials and Methods

In previous years, we carry out the on-line detection of pharmacologically active substances from a hydro-ethanolic extract of *Stevia rebaudiana* Bertoni (Asteraceae). This system was based on liquid chromatography (through MPLC or HPLC techniques) coupled to biological detection using perifused or perfused organs. In summary:**[**1]

Organs were obtained from Sprague-Dawley rats (weight: 250–300 g). They bred at the animal facilities of the University of La Laguna. They were fed with food and water *ad libitum*. Their use was authorized by the Ethical Committee (CEIBA) of the University of La Laguna.

Stevia rebaudiana Bertoni was collected in San Rafael (Alto Paraná, Paraguay), it's a perennial herb with sweetening and, in traditional medicine, medicinal properties. The aerial parts of the plant were air-dried and ground. Three extractions in reflux for 1 h were made with a mixture of ethanol to water (7:3). The extract was then filtered, evaporated under reduced pressure and then freeze-dried (final extract mass: 19.85 g).

Fractions of extract were dissolved in 1 mL of Krebs-HEPES solution and were subjected to chromatographic separation in Sepahdex G10 with Krebs-HEPES buffer/solution at 37° as eluent (a suitable physiological medium which allowed the organs to be kept alive). Isocratic conditions were maintained, the elution rate was 1 mL/min. This chromatographic separation was directly coupled to one superfused organ cascade as a quadruple biosensor (with aorta ring chain, tracheal ring chain, a 2 cm portion of the last part of the rat ileum, and one 1 cm of prostatic portion of the vas deferens mounted in a classic organ bath system). We measured their contractile response, having previously characterized the response of the organs with injecting a Krebs-HEPES solution containing 10 μM of well-known drugs like acetylcholine, noradrenaline, serotonin, and adrenaline. This system allowed us to separate the components present in the hydro-ethanolic extract and determine an active fraction. The active fraction was dried under pressure in a rotary evaporator and then structurally studied by mass spectrometry.

3. Results [1]

Direct injection of the hydro-ethanolic extract into the system at a concentration of 10 mg/mL^{-1} produced contraction of all four organs. At a higher concentration, 100 mg/mL^{-1}, these effects varied for three types of organs: (1) At higher concentration, the aortic rings experienced a small contraction, and then this contraction was followed by a long relaxation. (2) The contractile response of the prostatic portion of the vas deferens was briefer at the higher concentration and no subsequent relaxation occurred. (3) At 100 mg/mL^{-1}, contraction of the ileum was stronger, and it was followed by a prolonged relaxation. (4) The response in the tracheal rings was similar at both concentrations.

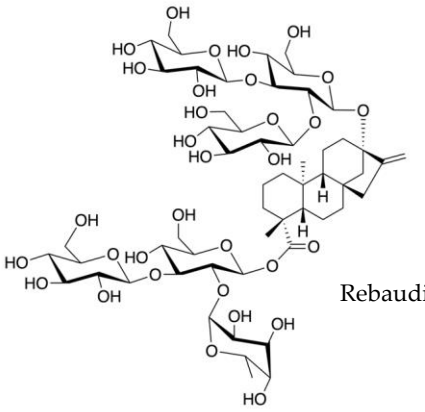
In another experiment, 1 mL of extract dissolved in Krebs-HEPES solution (extract concentration: 100 mg/mL^{-1}) was injected into the chromatographic separation system in Sephadex G-10 coupled directly to superfused organ cascade as a quadruple biosensor. This study allowed us to establish different fractions according to their absorbance at 254 nm, the contractile action produced on the organs and the elution time of the column. This last separation method was used to identify an active fraction that produced muscle contraction in the ileum tissue. This fraction did not absorb light with a wavelength of 254 nm and was detected 70 min after the start of separation on the column.

We repeated the chromatographic separation process under the same conditions but in the absence of superfused organ cascade as a quadruple biosensor. We separated and collected the fraction that begins its elution 70 min after the extract was injected into the separation system. Thus, the active fraction could be isolated and chemically studied.

The active fraction was studied by mass spectrometry. It showed a high degree of purity and rebaudioside N was the major compound. Our study allowed us to identify rebudioside N as the compound responsible for the contractile activity detected.

4. Discussion

Firstly, this research demonstrated the effectiveness of the proposed direct coupling method. This technique enabled us to easily identify the fraction that showed contractile activity. And it allowed to identify the contractile activity of a minor compound in the plant species *Stevia rebaudiana* Bertoni, the natural product rebaudioside N (see Figure 1).



Rebaudioside N

Figure 1. Chemical structure of rebaudioside N. It is a minority stevioside, one type of diterpene glycosides, in the *Stevia rebaudiana* Bertoni [1].

The buffer solution and chromatographic eluent (Krebs-HEPES solution) used is physiological and aqueous. It is free of toxicity and environmental bioaccumulation. These separation and working conditions comply with the principles of Green Chemistry.

This chromatographic separation directly coupled to one superfused organ cascade as a quadruple biosensor reduced the number of animals slaughtered, investigation time and the expenses associated with such studies.

Different types of standard analytical detectors can be inserted between the column and the organ preparation to provide additional information. Detectors can also be placed at the end of the column when it is not directly coupled to a living organ biosensor system. In this way, chemical properties can be studied in parallel, for example by coupling to mass spectrometry or other spectroscopic methods for rapid and easy identification of chemical compounds.

In addition, we describe uses and perspectives in bioorganic chemistry of coupling MPLC and HPLC to studies of living tissues or organs in the pre-characterization processes of new bioactive compounds present in mixtures derived from chemical synthesis (for example, combinatorial chemistry) or from extracts of natural origin:

1. For all the reasons stated here, this system for the pre-characterization of drugs allows the on-line detection of pharmacologically active substances in hydrosoluble mixtures from vegetal extracts or chemical synthesis.
2. Other organs or tissues may be included in the perifused or perfused system, thus expanding the pharmacological profile to be studied.
3. This system can characterize a range of drug activities, both the acute activity and the toxicity of the eluted substances.
4. Contractile activity studies implicate mechanisms regulated by receptors, ionic channels, contractile proteins, and second messengers. The on-line detection system will be useful for QSAR studies and the search for new drugs from a lead drug detected following the methodology described here.

5. Conclusions

The results show that a direct combination of liquid chromatography with perfusion systems of isolated organs may be a powerful tool to facilite the pharmacological characterization of active compounds in mixtures.

Not only from a time perspective, it allows saving energy, avoiding the use of toxic organic solvents, reducing the number of animals slaughtered and lowering costs. It is a methodology of interest for economically disadvantaged countries and for moving towards new methods that follow the principles of Green Chemistry.

I end the conclusions with the same phrase that closed the reference article of this full file text and published by our group: … "*Thus, this approach opens new and exciting possibilities in the field of drug research*" [1].

The uses and perspectives summarized here will be expanded and explained to form a small part of a future review article that will be submitted for evaluation and publication in a few months.

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Institutional Review Board Statement: This study was authorized by the Ethical Committee at the University of La Laguna (CEIBA) and was in accordance with ARRIVE (McGrath and Lilley 2015) and European Union guidelines (86/609/EEC).

Informed Consent Statement: This study was authorized by the Ethical Committee at the University of La Laguna (CEIBA).

Data Availability Statement: The data presented in this study are available in the reference [1].

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Conflicts of Interest: The author declares no conflict of interest.

Reference

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