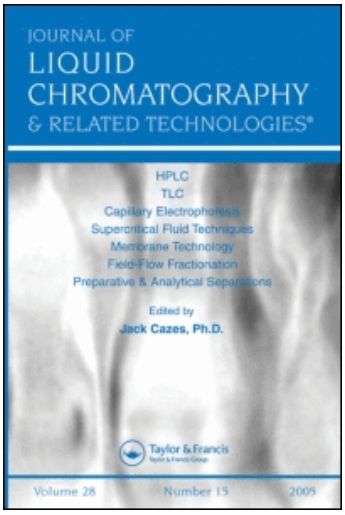

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Hazeyama, Y. , Valko, W. J. , Ravert, H. T. , Heindel, N. D. and Moretti, R. L.(1982) 'On Line Detection of Plasma Borne Vasoconstrictor by the Use of High Pressure Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 5: 9, 1749 — 1762
To link to this Article: DOI: 10.1080/01483918208067610
URL: http://dx.doi.org/10.1080/01483918208067610

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(9), 1749-1762 (1982)

ON LINE DETECTION OF PLASMA BORNE VASOCONSTRICTOR BY THE USE OF HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

An on line detection system for a plasma borne vasoconstrictor was developed using a rat heart bioassay and a reversed-phase high pressure liquid chromatograph (HPLC). Partially purified plasma borne vasoconstrictor, which is yet to be characterized, was fractionated by HPLC, and the output from the unit was introduced into the rat heart bioassay system directly. The on line HPLC-rat heart bioassay system detected the active fraction from approximately 20 substances in the partially purified plasma. This system enabled rapid and reproducible identification of the active vasoconstrictor in plasma.

INTRODUCTION

The existence of a vasoconstrictor component in blood plasma has been suggested but never proven (1, 2,3). This component apparently is not a commonly known vasoconstrictor like norepinephrine or angiotensin (5,6). One reason for the difficulty in

isolating and characterizing this plasma component may be due to the fact that the design of a separation process which is coupled to a meaningful biological assay is quite difficult for this type of material. Such a separation coupled to a biological assay is a requisite for successful purification of components of complex biological mixtures.

For the isolation of this vasoconstrictor component from human plasma, Bohr employed Sephadex gel G-15, and collected the elution fractions, and tested their biological activities using arterial strips from rats and rabbits (7,8). Moretti and others recently reported a similar substance and partially purified it using chemical procedures and thin layer chromatography (9-11). However, the purification methods utilized by the above-mentioned researchers are not effective and often require tedious preparation of the sample for the bioassay (1,7).

We have developed a system for the above purpose in which the powerful separation capability of reversed-phase HPLC is directly incorporated into a rat heart bioassay. In this system the biological activity of the components eluted from the chromatography column can be directly determined by a rat heart perfusion bioassay.

One of the properties of the plasma borne vasoconstrictor is the production of vasoconstrictions in the coronary, skeletal muscle, and perhaps renal circulations (12). Because many known vasoconstrictors do not produce vasoconstriction in the coronary circulation, even though they do produce constrictions in other organs, and because the preparation of the rat heart bioassay is relatively easy, we used the rat heart as the detector organ (13-15).

MATERIALS

As for the mobile phase, one liter of glass distilled water was filtered with millipore filter (Millipore Corp.) under faucet vacuum and then degassed for approximately 20 minutes by faucet vacuum. A similar treatment was done for the methanol used as the elution solution. HPLC grade methanol (J.T. Baker) was used for chromatography and all other chemicals were ACS reagent grade. The Tyrode's solution contains 151 mM NaCl, 4.4 mm KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.39 mM Na_2HPO_4, 13.2 mM $NaHCO_3$, 6.2 mM glucose, pH 7.4, 37^{o}C, saturated with 95% O_2, 5% CO_2 in distilled water. In order to establish iso-osmolarity at the perfusion

inlet to the coronary circulation, the osmolarity of Tyrode's solution is increased to approximately 10% above that of normal Tyrode's solution.

A Cole Parmer peristaltic pump (Master Flex - 7562) provided constant perfusion of Tyrode's solution to the coronary circulation. The pressure changes were detected by a Statham pressure transducer (PB-23) and recorded on a dual channel recorder (Versagraph 885).

The HPLC used in the experiment was a Perkin Elmer dual pump (Series 2/2) HPLC with a 4.6 mm x 25 cm reversed-phase column (Perkin Elmer C-18) and a syringe loaded injection valve (Rheodyne Model 7125 with a 20 µl loop). A variable wave length UV-visible detector (Perkin Elmer LC-75) was set at 254 nm for optimum peak detection. Chromatograms were recorded on the dual channel strip chart recorder.

METHODS

Extraction The original chemical extraction procedure for the vasoconstrictor substance was established by Moretti et al. (2) and modified for use in this study. One thousand ml of human platelets were obtained from a local blood bank. After the adjust-

ment of the pH to 3.5 by acetic acid they were washed with 2000 ml of ethyl ether in order to remove neutral lipids. The ether layer was discarded. After repeating the ether wash, the pH was adjusted to 2.5 with hydrochloric acid and the solution saturated with NaCl. An equal volume of chloroform: methanol (2:1, volume) was added to the aqueous phase to precipitate proteins. The solution was centrifuged and the resulting chloroform: methanol layer was removed by a 100 ml syringe. The chloroform: methanol extraction was repeated three times, and the fractions were combined and dried in vacuo. To the residue was added ether: methanol: water (1:1:1, pH = 3.0, adjusted by HCl). The aqueous phase was collected, dried, dissolved in methanol and stored in a cold room. Since the quantity of the vasoactive substance extracted is unknown, we designated this extract as 1000 ml equivalent of plasma (ml eq.).

Chromatography and Bioassay One thousand ml eq. of crude plasma extract was condensed to 1 ml volume in methanol. In order to evaluate the quality of vasoconstrictor extraction 10 µl of the extract was injected onto the reversed-phase column and eluted with a 10% methanol-water mobile phase at a rate of

1 - 2 ml/min. These prebioassay chromatograms were obtained without using the rat heart.

The coronary circulation of excised rat heart was connected to the perfusion system shown in figure 1. The heart was perfused with oxygenated Tyrode's solution at constant flow (9 ml/min). Before starting any measurements, the heart was perfused for 20 minutes to remove residual plasma.

One thousand ml eq. of crude extract was completely dried, freed from methanol, and dissolved in 1 ml of distilled water. Ten µl of this methanol-free extract solution was injected onto the reversed

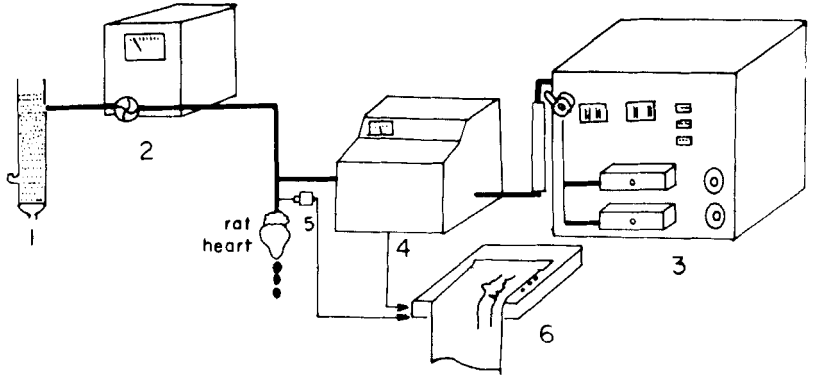

Figure 1. A schematic presentation of a rat heart bioassay system utilizing HPLC. Bubbled Tyrode's solution 38°C (1) was perfused into a rat heart by a peristaltic pump (2). The effluent from the HPLC (3) was also infused into the heart. The perfusion pressure measured by a pressure transducer (5) and UV absorption measured by a UV-visible detector (4) were recorded on a dual channel recorder (6).

phase column and eluted with distilled water at 1 ml/min. The effluent was directly infused into the perfused heart. The pressure change in the coronary circulation resulting from the eluting substance was detected by the pressure transducer, and UV absorption due to the eluting substance was recorded simultaneously.

RESULTS AND DISCUSSION

Evaluation of Chemical Extraction The vasoconstrictor component of plasma extract was separated by reversed phase HPLC column. A solvent system of 10% methanol and 90% water produced the most efficient separation. A representative chromatogram obtained by the above described methodology is shown in figure 2. As shown in the figure, two major peaks are found in the early period of elution and approximately 15 peaks were detected, indicating that the crude plasma extract contains at least 15 substances. Some variability was observed in the profile of the chromatogram among different extract preparations; however, the major features of the chromatogram were consistent. If the features were substantially different from the ones shown in figure 2, that extraction sample was discarded.

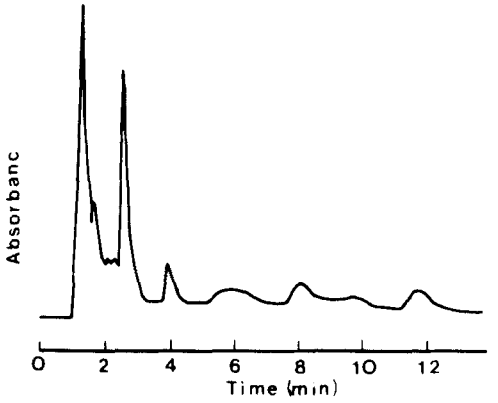

Figure 2. Chromatography of the crude plasma extract using 10% methanol and 90% water. Experimental details as described in Methods.

Although a solvent system of 10% methanol and 90% water produced the most efficient separation, because of the toxic nature of methanol this solvent system was not used for the bioassay experiment. Since a pure water solvent system produced a similar elution pattern to the methanol-water system, we used pure water for the HPLC-bioassay system.

Rat heart-HPLC Bioassay In order to determine which peaks represented the vasoactive fractions we directly infused elution solution from the reversed phase HPLC (mobile phase H_2O) to the perfusing line of the coronary circulation of the rat heart. A chromatogram and a pressure response from a typical experiment are shown in figure 3. In this particular

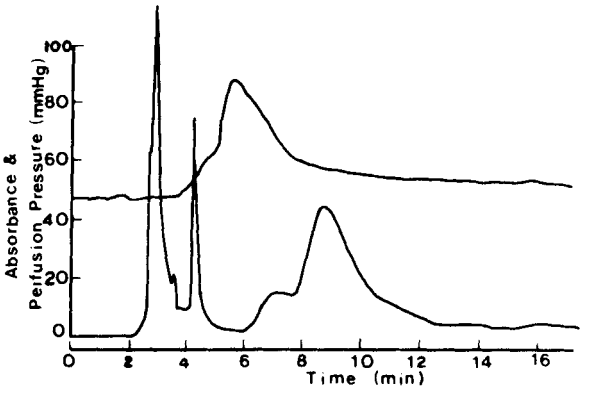

Figure 3. A typical bioassay experiment showing the pressure response (upper trace) and HPLC chromatogram (lower trace) of the crude plasma extract. Experimental details as described in Methods.

experiment, the total flow was 9 ml/min (8 ml/min of Tyrode's solution and 1 ml/min of elution solution). Although there was some possible development of ischemic conditions, the heart functioned as an assay organ throughout the course of the experiment.

Three groups of peaks were observed in this particular chromatogram when 10 µl (10 ml eq.) of plasma extract was injected. The perfusion pressure before constriction was 46 mmHg. With the elution of the various fractions, a pressure increase to a maximum of 86 mmHg was observed. This level of pressure change was sufficient to reliably locate the active component in the HPLC elution profile. Although only one peak is observed in the pressure

tracing, a close inspection of the pressure tracing shows a stepwise increase in the perfusion pressure. The stepwise increase in the pressure indicates the existence of more than one active substance in the plasma extract. Since the recorder pens for the pressure and chromatogram are one half minute out of phase, the first group of peaks on the chromatogram is related to the initial pressure increase and the second large peak to the second pressure increase. Apparently the third group of peaks yields no pressure change.

We performed six perfusion experiments using plasma extracts obtained from different extract preparations. The pressure peak was produced at 5.13 ± 0.26 min (S.D., n = 6). This information enabled us to determine the particular peak in the chromatogram which is related to the coronary vasoconstriction. Similarly, we could conclude that the first small pressure increase was produced by the substance which was eluted right after the void volume.

While the first and second peaks on the chromatogram eluted relatively quickly, the pressure response lasted approximately 5 minutes. The slow return toward preconstriction pressure level is pos-

sibly intrinsic in the nature of the coronary circulation system. One may note that the perfusion pressure after the constriction never returned to the preconstriction pressure. This effect may be attributed to the absence of the stabilizing influence of large-molecule substances (i.e. proteins) lacking in the perfusate. Due to edema formation in the heart, response to the plasma fraction gradually diminished. In general the heart functioned as an assay organ for approximately two hours.

If the HPLC effluent fractions were collected from the outlet of the HPLC column and assayed subsequently for biological activity, 30 to 50 runs might be required to locate the active component. Since each individual biological test by direct injection to the coronary circulation system (instead of using this on line assay system) takes 5 minutes, at least 150 minutes of testing time is required to do the task. In addition to this testing time, each fraction has to be collected, dried and redissolved into a small volume of solution for the bioassay. Because each additional step in the collection and purification process causes additional losses of the compound, a larger amount of sample has to be extracted to start with. Therefore, the

total time required to identify the active fraction by individual testing methods becomes enormous. The same work can be done on-line within 15 minutes in the system reported herein. This HPLC and rat heart bioassay system saves considerable time, effort and expense without compromising accuracy in the detection of the active fraction of plasma borne vasoconstrictor.

Finally studies to establish the true identity of the two vasoconstrictive factors are underway at present. The preliminary results suggest that the vasoactive substances are heat stable, small molecules and are not commonly known vasoactive substances; however, the characterization of the exact nature of the substance will require further research.

ACKNOWLEDGMENTS

This research was supported in part by the Dorothy Rider Pool Health Care Trust

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