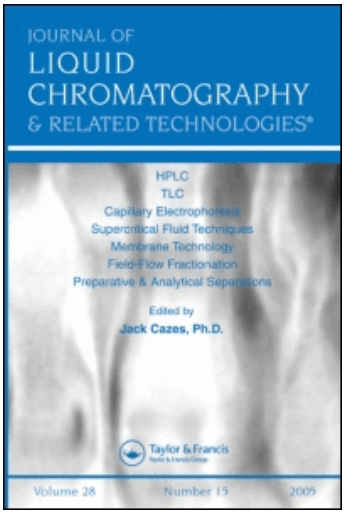

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Determination of Rhein Anthrone in Caecal Content of Rats Using Reversed Phase HPLC

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DETERMINATION OF RHEIN ANTHRONE IN CAECAL CONTENT OF RATS USING REVERSED PHASE HPLC

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ABSTRACT

A sensitive spectrofluorimetric method is described to determine small quantities of rhein anthrone in complex biological systems as caecum content and faeces. The method is based on the stability and fluorescent properties of anthrone molecules in borax solution and combines the sensitivity of spectrofluorimetric methods with the resolution power of liquid chromatography.

After extraction of the faeces with a solution of sodium tetraborate and ascorbic acid in water and centrifugation, an aliquot of the supernatant is injected on a Sephadex-G25 precolumn. After rejection of the first fractions (fluorophors present in caecal content), rhein anthrone is collected on an RP-C18 column and eluted with an acetonitrile-borax buffer gradient. The anthrone is quantified using a fluorescence HPLC monitor.

INTRODUCTION

The dihydroxy-derivatives of anthrone (dihydroxy-9,10-dihydro-9-oxoanthracene) are biologically active substances which frequently are used as laxatives or as antipsoriasis drugs in human therapy.

In the latter case mainly anthralin (dithranol) is employed as a topical agent (0.1-1 %) in pastes and ointments. These are applied to the lesions and can be

combined in well-defined programs with exposure to UV and other topical products as coal tar and corticosteroids (1-6).

In constipation therapy with anthranoid laxatives however some anthrone-derivatives like dianthrone O-glycosides (e.g. sennosides) or anthrone C-glycosides (e.g. cascarosides) are preferred since the oral administration of free anthrones would induce mucosal irritation of the stomach (7) with emetic side-effects (8). These glycosides represent true prodrugs which travel through the gastro-intestinal tract untill they are split by the bacterial microflora of the colon into the anthrone aglycon which exerts its laxative action (8).

Because these anthrones are labile molecules which are easily transformed to anthraquinones, dianthrones and some brown substances (9,10) (figure 1), difficulties may be expected when investigating the pharmacological and biochemical properties of these substances. First of all the individual activity of the different decomposition products should be examined. Secondly, since anthrone concentrations tend to drop sharply in aqueous solutions, problems will arise when using anthrones in biological and biochemical experiments.

In order to correlate the concentration of the different compounds with the test responses, some efforts have been made to analyze quantitatively the conversion of dithranol (9, 11-13) or rhein anthrone (10) to their decomposition products by using spectroscopic or HPLC methods. The latter technique has also become the first choice in the analysis of galenic preparations of anthralin (14-18), since it was proven (19) that quantification of breakdown products can only be made after separation of the compounds. However as it was pointed out (15,16) some important precautions (e.g. use of low-actinic glassware, deaeration of all solvents) should be taken in order to eliminate degradation during sample preparation and analysis.

Being interested in the mechanism of action of anthranoid laxatives, we investigated the degradation and binding of rhein anthrone to caecal content of rats. As an alternative to the current methods for the analysis of anthrones, we developed a technique which takes advantage of the stability and fluorescent properties of anthrones in borax solutions (20). In this way the labile rhein anthrone could be extracted from caecal content and separated by chromatography from other fluorescent compounds without deterioration.

EXPERIMENTAL

Chemicals

Sodium tetraborate decahydrate (borax, Janssen Chimica) and ascorbic acid (Merck) were analytical-reagent grade products. Acetonitrile (HPLC grade) was

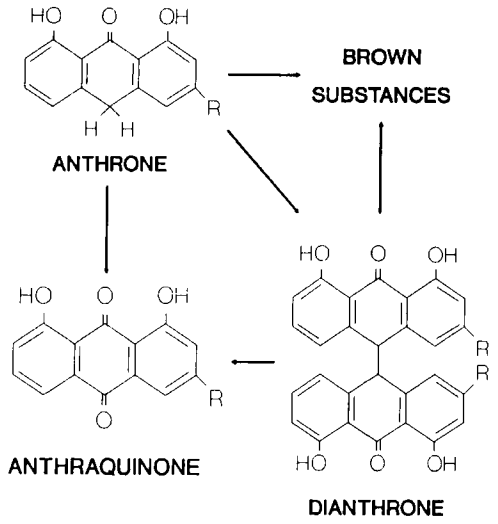

Figure 1 : Degradation of anthrones to anthraquinones, dianthrones and some brown substances (R = COOH : rhein, R = H : chrysazin).

purchased from Rathburn. All aqueous solutions and extracts were filtered through a 0.45 μm filter (Spartan 30/B, S & S). The borax-ascorbic acid solutions were freshly prepared before use.

Rhein antraquinone (1,8-dihydroxy-3-carboxyanthraquinone) was prepared according to Bellaart (21), starting from aloin (MacFarlan Smith Ltd). Rhein anthrone (1,8-dihydroxy-3-carboxy-9-anthrone) was synthetized according to de Witte and Lemli (22). The purity was 94 %, as was proven with [^{14}C]rhein anthrone (23). The anthrone was stored under dry and inert conditions, protected from light.

Apparatus and Columns

The chromatographic system consisted of two pumps (P-500) connected with a gradient programmer (GP-250) (FPLC, Pharmacia), equipped with an automatic injector (ACT-100, Pharmacia, RSD on injection of a 50 μL sample : 0.22 %) with a 50 μL loop, a Sephadex G25 precolumn (7 cm x 7.0 mm I.D., particle size : superfine, Pharmacia) linked by a valve system (SRV-3, Pharmacia) with a reversed phase C18 column (PepRPC HR 5/5, 5.3 cm x 7.0 mm I.D., particle size : 5 μm, Pharmacia) and a Shimadzu model RF-535 spectrofluorimetric detector, equipped with a 12 μL flow cell. The fluorimeter monochromators were set at 405 nm for excitation and 515 nm for emission. A Shimadzu integrator (C-R3A) was employed for peak recording and surface integrating.

Chromatography Conditions

Analysis of rhein anthrone in caecal extract : 50 μL of caecal extract was injected on the Sephadex G25 precolumn and isocratically eluted at room temperature at a flow rate of 0.15 mL/min with a mixture of 2 % (g/v) borax and 1.3 % (g/v) ascorbic acid in bidistilled water (= borax elution buffer, pH : 8). The first 21 min fraction (3.15 mL) was rejected (waste), followed by adjusting valve 2 to pass the mobile phase over the RP C18 column. Fourty-two minutes after injection the position of valve 1 was changed in order to by-pass the Sephadex precolumn.

Subsequently the compounds collected on the top of the RP-C18 column were directly eluted at room temperature (flow rate of 0.5 mL/min) with a gradient of acetonitrile (solvent a) and the borax elution buffer (solvent b). The program consisted of the following data : 0-3 min (0-25 % solvent a), 3-10 min (25-45 % solvent a), 10-15 min (0 % solvent a). The set-up of the two colums is shown in figure 2.

Separation on the Sephadex precolumn : in order to verify the reproducibility of the retention times of the anthranoids and fluorophors present in the caecal extract on the Sephadex G25 precolumn, each day a 50 μL sample of rhein anthrone (0.5 μg/mL), rhein anthraquinone (50 μg/mL) and caecal extract (blanco), or a combination of these, was injected. The fluorescence detector was directly connected with the waste exit of valve 2 (see figure 2) to monitor the elution of the different fluorescent compounds.

Calibration Graphs, Precision and Recovery of Rhein Anthrone

The calibration graph for rhein anthrone has been obtained by plotting the peak area against concentration (0.1, 0.3, 1.0, 2.5 and 5.0 μg/mL) of anthrone injected on the Sephadex precolumn, followed by quantification after elution from the RP C18 column. The different concentrations were prepared by dissolving rhein anthrone in caecal extract (see further).

The same dilutions of rhein anthrone were made in borax extraction buffer (see further). By adjustment of valves 1 and 2 (see figure 2) 50 μL was injected directly on the RP C18 column without Sephadex clean-up. By comparing the calibration graph obtained in this way with the former, the recovery of rhein anthrone from the Sephadex precolumn was calculated.

Assay precision was evaluated by calculating the coefficient of variation (CV) of results obtained by repeated analysis (n = 5, injection on the Sephadex precolumn) of a 1 μg/mL solution, and by normalisation of the values of the calibration graphs.

Extraction Procedure

An amount of caecal content (0.5-4 g) was vigorously shaken for 10 minutes with 20 mL of a 2 % (g/v) borax-0.5 % ascorbic acid mixture in water (=borax

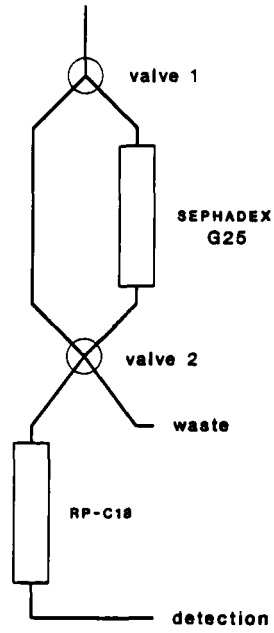

Figure 2 : Set-up of the RP-C18 column and the Sephadex G25 precolumn.

extraction buffer). After centrifugation (50.000 g, 10 min) to precipitate caecal debris, the supernatant was decanted and diluted with borax extraction buffer to 25 mL. An aliquot (50 μL) of the caecal extract was analyzed. The caecal debris was reprocessed for a second and eventually a third extraction.

Analysis of Caecal Content Spiked with Rhein Anthrone

In order to analyze the degradation and binding properties of rhein anthrone, 100 μg, 1 or 4 mg rhein anthrone was dissolved in 1 mL of a freshly prepared mixture of 2 % Na_2CO_3 and 0.5 % ascorbic acid in water. Immediately before injection the alkaline solution was diluted with 1 mL of an appropriate acetic acid solution to adjust the pH to 7.8. A sample was taken and diluted to a proper volume of borax extraction buffer to determine the rhein anthrone content. The final rhein anthrone solution (2 mL) was injected intracaecally (in vivo method) in anesthetized rats or in freshly isolated caecal content (in vitro method) and gently mixed. In the latter case the caecal content of killed rats was collected in capped vials which were incubated at 37°C. The amount of extractable rhein anthrone was analyzed after 30 minutes of contact with the caecal content (n = 3).

RESULTS and DISCUSSION

The fluorimetric method goes back to Lane (20), who proved the fluorescent properties and stabilization of anthrones in aqueous sodium tetraborate solutions. However since no purification prior to the fluorescence measurements was provided, problems could arise with concentrated extracts of complex biological systems. Especially caecal extracts showed high quenching characteristics and autofluorescence, which interfered dramatically with the analysis of low concentrations of rhein anthrone in borax solutions. Therefore a HPLC method was developped which combines the advantages of the fluorescent method with the separation power of liquid chromatography.

The original method of Lane (20) used ß-mercapto-ethanol as an anti-oxidant, which in the present study was replaced by the non-pungent and non-toxic ascorbic acid. This compound also lowered the pH of the aqueous borax solution (2 % , pH = 9.2) in order to protect the RP C18 column against rapid degradation. In these circumstances rhein anthraquinone was not reduced to rhein anthrone by means of the present anti-oxidans.

The clean-up with a Sephadex G25 precolumn was a necessary step in the analysis of rhein anthrone present in caecal extracts, since no satisfying separation of some fluorophors present in caecum content and rhein anthrone could be achieved on a RP C18 column. Figure 3 shows the elution of the fluorescent caecal components, rhein anthraquinone (50 µL/mL) and rhein anthrone (5 µL/mL).

The retention times (respectively 8.1, 17.8 and 28.2 minutes) were highly reproducible, as proved by the day-to-day analysis. In this way no monitoring of the elution of the different components from the Sephadex precolumn was necessary and the valve position could be routinely switched after a period of 21 min. This manipulation changed the route of the mobile phase and applied a part of rhein anthraquinone (when present) and the entire amount of injected rhein anthrone on the top of the RP C18 column (figure 3). Afterwards the two compounds were chromatographed with a gradient of borax buffer and acetonitrile (retention times : rhein anthrone 7.3 min, rhein anthraquinone 9.2 min).

As only a part of rhein anthraquinone (when present) injected on the Sephadex precolumn was collected on the reversed phase column, the method with a Sephadex clean-up was only workable to analyze rhein anthrone. However in other analyses the Sephadex precolumn conditions might well be unnecessary which offers the opportunity to inject the compounds directly on a RP column.

The respons with rhein anthrone dissolved in caecal extract proved to be linear up to 5 µg/mL ($r = 0.999$) after injection of 50 µL on the Sephadex precolumn. The recovery, i.e. the amount of rhein anthrone detected after passage on Sephadex and

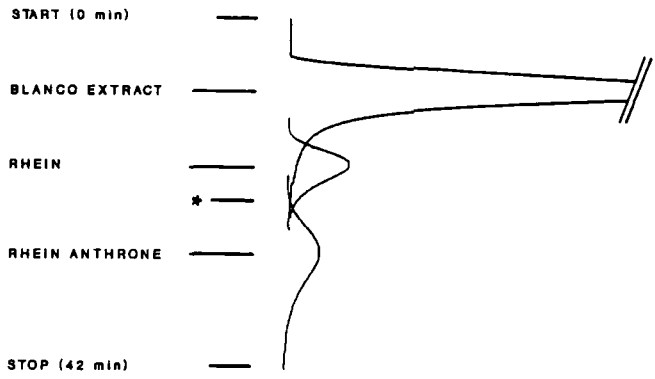

Figure 3 : Superposition of the chromatograms obtained after elution of rhein anthraquinone (50 μg/mL), rhein anthrone (0.5 μg/mL) or fluorescent components present in caecal extract on a Sephadex G25 precolumn. (* : adjustment of valve 2 after 21 minutes (see figure 2)).

elution of the RP column in comparison with the amount detected after direct injection on the RP column, was 93.1 ± 2.3 % (mean ± SD, n = 5).

The coefficient of variation (CV) of results obtained by repeated analysis of a 1 μg/mL solution was 3.5 %. After normalisation of the data of the calibration graphs, the CV was 2.7 % in the case where the anthrone was injected directly on the RP column and 7.0 % in the case where the anthrone passed by the Sephadex precolumn.

The detection limit proved to be 1 ng (on column), which corresponds with a total amount of 0.5 μg rhein anthrone extracted from caecal content. This quantity was equivalent to more than five times the respons of a blanco injection on the Sephadex precolumn followed by elution of the RP column (see figure 4).

The extracts of rhein anthrone proved to be stable for at least two hours at room temperature and at least 18 hours at 4 °C.

The recovery of rhein anthrone in vivo and in vitro from caecal content is shown in figure 5. After 30 minutes of contact with caecal content, only a small fraction (ca. 6-7 %) of an injection of 100 μg rhein anthrone is extracted. As further extractions did not improve substantially the yield, it is proven that the compound is bound to some caecal components and/or degraded to non-anthrone substances. Furthermore it is obvious that the more is injected the more is recovered, but also the

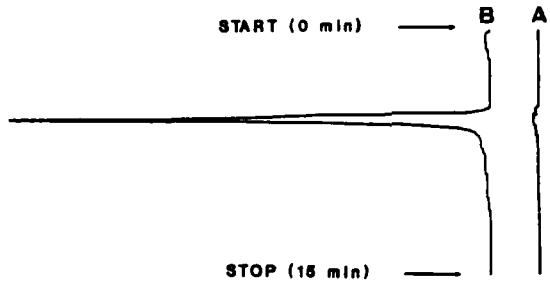

Figure 4 : Chromatograms obtained by injecting blanco caecal extract (a) and caecal extract spiked with rhein anthrone (0.5 μg/mL) (b) on a Sephadex G25 precolum, followed by fluorimetric detection after elution of the RP C18 column.

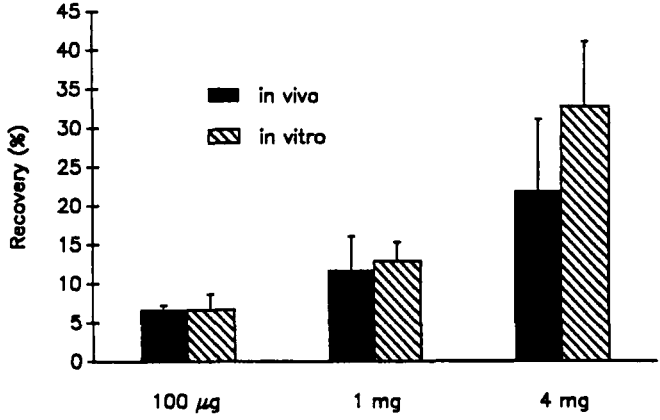

Figure 5 : Recovery of rhein anthrone in vivo and in vitro from caecal content after 30 minutes, expressed as percentage of the injected dose (100 μg, 1 mg, 4 mg). The mean and standard deviation is shown (n = 3).

difference between the in vivo and in vitro method becomes apparent. This phenomenon is probably due to the possibility of resorption in vivo when rhein anthrone is injected in the caecum.

The data prove that rhein anthrone behaves in caecal content as a labile compound, so likely only a small free and not deteriorated fraction of the anthrone injected in the caecum is responsible for the laxative action.

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