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# USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO SEPARATE VERY SMALL QUANTITIES OF RADIO-LABELLED METABOLITES FROM BIOLOGICAL TISSUE

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## SUMMARY

In order to study the absorption, metabolism and tissue distribution of chemicals in cattle ticks *Amblyomma variegatum*, a method of separating and recovering metabolites in picogram quantities was required. A high-performance liquid chromatographic system has been investigated for this purpose, using tritium-labelled amitraz and some potential metabolites. Separation was carried out on Nucleosil 5 $\mu$m $NO_2$ bonded phase. Interchangeable pre-columns were used to extend the range of compounds which could be separated in one run and to keep the main column free of radioactive contamination.

Amitraz, 2,4-dimethylaniline and 2,4-dimethylformanilide were eluted from the column quantitatively without the addition of unlabelled carrier, down to quantities of 0.75 pg, 0.78 pg and 1.60 pg, respectively.

Apparatus for automated collection of the column eluate in scintillation vials and a method for data processing are described.

## INTRODUCTION

In our laboratories we are concerned with measuring the absorption, tissue distribution and metabolism of various chemical compounds within cattle ticks. The ticks being studied are *Amblyomma variegatum* which in the unfed adult male state measure approximately 7 mm × 4 mm and weigh about 20 mg. Dissection and recovery of tissues is a lengthy and difficult process which prohibits using large numbers of ticks in order to obtain appreciable quantities of tissue. Hence metabolite separations must be carried out on very small quantities of tissue, with only picogram quantities of compounds being estimated. Most compounds being studied are tritium-labelled for cheapness and in order to obtain the high specific activities required.

Thin-layer chromatography (TLC) followed by scraping of spots and scintillation counting is often used for metabolism studies but it is difficult to visualise the spots on the plate with low levels of tritium[1] and recovery from the absorbent is not always quantitative[2,3]. Radio gas chromatography systems have been described with either on-line counting via a proportional counter[4,5] or off-line counting by combus-

tion[6] or pyrolysis[7] of the gas chromatographic (GC) effluent and collection of the products in scintillation vials. GC techniques are limited to the analysis of volatile metabolites and difficulties can arise through contamination of the column by involatile materials[8].

High-performance liquid chromatography (HPLC) offers many advantages for metabolism studies, particularly in the wide range of chemical types that can be separated. Methods are available for monitoring the radioactivity in the column effluent directly, either by passing through a plastic scintillator[9] or by mixing with scintillant solution and using a flow cell in a scintillation counter[10]. "On-stream" counting methods are not suitable for samples with very low activities however, due to short residence times within the counter. The system described here uses off-line monitoring of the liquid chromatographic (LC) effluent with fractions being collected in scintillation vials.

LC detectors are generally less sensitive than GC detectors[11,12] and it has not been adequately demonstrated that picogram quantities of compounds can be eluted from the LC columns without loss. In the present study, column losses and cross-contamination have been studied using the acaricide amitraz [1,5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triazapenta-1,4-diene] and two potential metabolites[13], 2,4-dimethylaniline (I) and 2,4-dimethylformanilide (II). These compounds had been prepared at high specific activities for other studies.

A two-part column system has been employed, consisting of a main column and interchangeable pre-column. The pre-column served two functions, to keep the main column free of radioactive contamination and to extend the range of compounds which could be separated in a single run. This latter point is illustrated by an additional, suspected, metabolite of amitraz, 4-amino-3-methylbenzoic acid (III) which is difficult to chromatograph with the other compounds, particularly I, since it is primarily acidic in character and acidic and basic compounds tend to have conflicting solvent requirements for good peak shapes[14,15].

## APPARATUS

The apparatus consisted of a Varian LC 8500 dual syringe pump system with gradient elution. Injection was by means of a pneumatically driven Valco 6-port valve fitted with a 50-$\mu$l loop. All injections were made by partially filling the loop with a 50-$\mu$l syringe.

Columns were 4 mm I.D. stainless steel packed with 5 $\mu$m Nucleosil $NO_2$ bonded phase. Lengths of 5 cm and 20 cm were employed, packed in our laboratories using a high-viscosity cyclohexanol–toluene (2:1) slurry[16].

Detection was by a Cecil Instruments CE 212 variable wavelength UV detector.

The column effluent was mixed with a toluene PPO/POPOP scintillant solution and collected in liquid scintillation vials using an ISCO Model 328 fraction collector. This instrument has the facility of operating a stop valve 0.8 sec before each bottle change. Two valve configurations were employed. Initially, two pneumatically operated slides valves (Durrum, Palo Alto, Calif., U.S.A.) were fitted as shown in Fig. 1. The valve in the LC effluent stream had a bore of 0.8 mm and a total internal volume of 8 $\mu$l. Pressure rating was 500 p.s.i. which was quite adequate to cope with

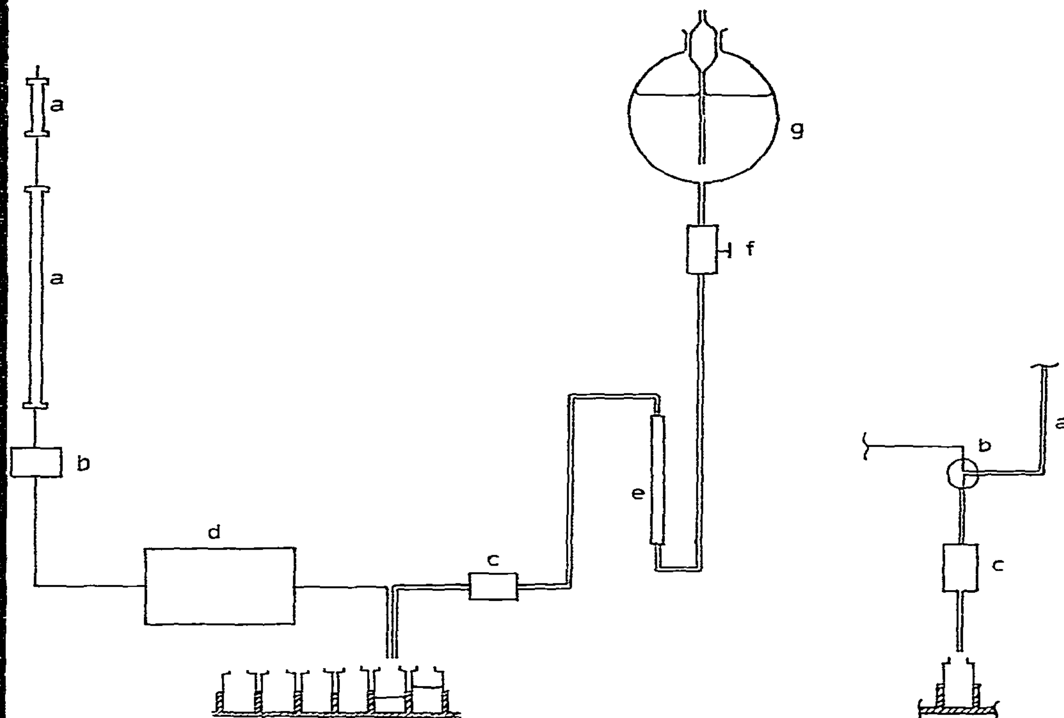


Fig. 1. Valve arrangement for automated collection of fractions and addition of scintillant mixture. a, Column; b, slider valve, 0.8 mm bore; c, slider valve, 1.6 mm bore; d, detector; e, flow-meter; f, needle valve; g, constant head solvent reservoir.

Fig. 2. Detail of simplified valve arrangement. a, 1/8 in. O.D. PTFE tubing; b, low-dead-volume tee; c, slider valve, 1.6 mm I.D.

the small pressure build up at the end of the column during bottle changes. It was necessary to fit this valve before the detector to prevent baseline disturbances due to flow and pressure changes inside the cell. No peak broadening was observed at peak widths down to 10 sec.

The simpler configuration shown in Fig. 2 proved equally effective. Scintillant and effluent stream were mixed in a low-dead-volume Kel-F tee and then passed into a single stop valve. During the 2 sec that the valve was shut during bottle changes, the pump forced the column effluent approximately 5 cm up the scintillant tube but this was immediately flushed through as the valve re-opened. This very simple relief mechanism prevented any disturbance of the baseline even at highest sensitivities.

## MATERIALS

All solvents were standard laboratory grade, distilled in glass prior to use.

Tritium-labelled amitraz and compounds I and II were labelled in the 5-position of the aromatic ring by exposing the iodo derivatives to tritium gas at ambient temperature over a palladium on barium carbonate catalyst in dimethylformamide

TABLE I

MAXIMUM SPECIFIC ACTIVITIES OF TRITIUM-LABELLED COMPOUNDS AVAILABLE

| *Compound* | *Specific activity (Ci/g)* |
|---|---|
| Amitraz | 120 |
| 2,4-Dimethylaniline (I) | 96 |
| 2,4-Dimethylformanilide (II) | 120 |

solution. The specific activities available, before dilution with unlabelled compounds, are given in Table I. Samples were purified by TLC immediately before use.

4-Amino-3-methylbenzoic acid was not tritium-labelled and was obtained from Aldrich (Milwaukee, Wisc., U.S.A.).

## PROCEDURES AND RESULTS

### *Chromatography*

As mentioned earlier, the separation was divided into two parts; amitraz and components I and II were separated on a column of Nucleosil $NO_2$ phase using a

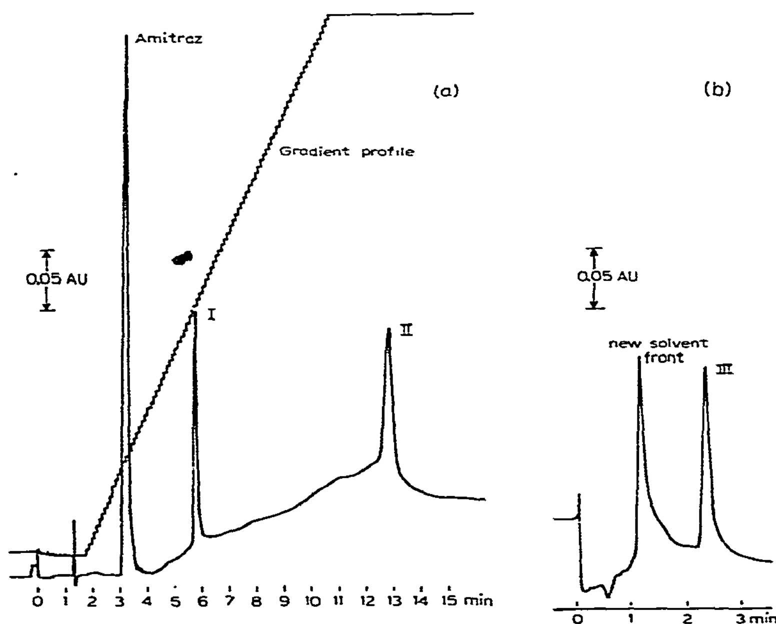


Fig. 3. (a) Chromatography of amitraz and some potential metabolites. Column, two sections, 50 mm × 4 mm and 200 mm × 4 mm, each packed with Nucleosil 5 μm $NO_2$ phase. Solvent, 10% ether in hexane + 0.3% triethylamine for 2 min then programmed at 10%/min to ether + 0.3% triethylamine. Flow-rate: 100 ml/h. Pressure, 3000 p.s.i. Temperature, ambient. Detection, UV at 280 nm, 0.5 a.u.f.s. Injection, 10 μl of diisopropyl ether containing amitraz, 200 μg/ml; I, 400 μg/ml; II, 1 mg/ml and III, at 50 μg/ml. (b) Elution of compound III from pre-column. Column, 50 mm × 4 mm. Solvent, ether + 0.5% acetic acid. Flow-rate, 60 ml/h. Pressure, 250 p.s.i. Temperature, ambient. Detection, UV at 300 nm, 0.5 a.u.f.s.

gradient of 10% ether in hexane to 100% ether, each solvent containing 0.3% triethylamine (see legend of Fig. 3a for the chromatography details).

Under these conditions III was retained on the pre-column and was subsequently eluted with 0.5% acetic acid in ether (Fig. 3b).

To save changing solvents in the pumps during each run, the second part of the chromatography was not performed immediately; after elution of the first group of compounds the pre-column was removed, securely capped and a fresh pre-column fitted. Initial conditions of 10% ether in hexane were restored by reverse gradient. Pre-columns were eluted in batches of ten and were finally flushed with methanol before re-use.

No losses of III occurred during the period the pre-columns were stored.

*Losses on the chromatography column*

Four solutions of unlabelled amitraz and compounds I and II at concentrations of 200 μg/ml, 2 μg/ml, 20 ng/ml and 200 pg/ml per component were prepared in diisopropyl ether and then spiked equally with tritiated compounds to the levels shown in Table II. A fifth solution was prepared containing the same quantities of tritiated materials but without any unlabelled compounds. Each solution also contained 1% dimethoxyethane to reduce absorption on the glass surfaces.

TABLE II

MINIMUM QUANTITIES OF EACH COMPONENT INJECTED ON TO THE COLUMN AND PERCENTAGE RECOVERIES AT THAT LEVEL

| *Compound* | *Level of radioactivity in each solution (nCi/ml)* | *Quantity injected on to column in solution without carrier (pg)* | *Recovery from solution without carrier** |
|---|---|---|---|
| Amitraz | 9.0 | 0.75 | 101% |
| I | 7.5 | 0.78 | 98% |
| II | 19.2 | 1.60 | 104% |

* Expressed as the percentage of the activity found in the 200 μg/ml carrier solution.

Duplicate 10-μl injections of each solution were made, starting with the highest concentration, using the gradient profile previously described. Between each different solution a blank injection and gradient run was performed to reduce any memory effects.

Quantities of each component injected on to the column therefore spanned six orders of magnitude through the range of solutions. The lowest quantities injected, *i.e.* from the solution without carrier, are given in Table II, together with the recoveries obtained at this level expressed as a percentage of the activities recovered from the highest concentration. Activities recovered at each level are shown graphically in Fig. 4.

*Cross-contamination*

This can be manifested in two ways, either by an increase in the radioactivity baseline during a run, following the elution of a highly active peak or by contamination of succeeding runs.

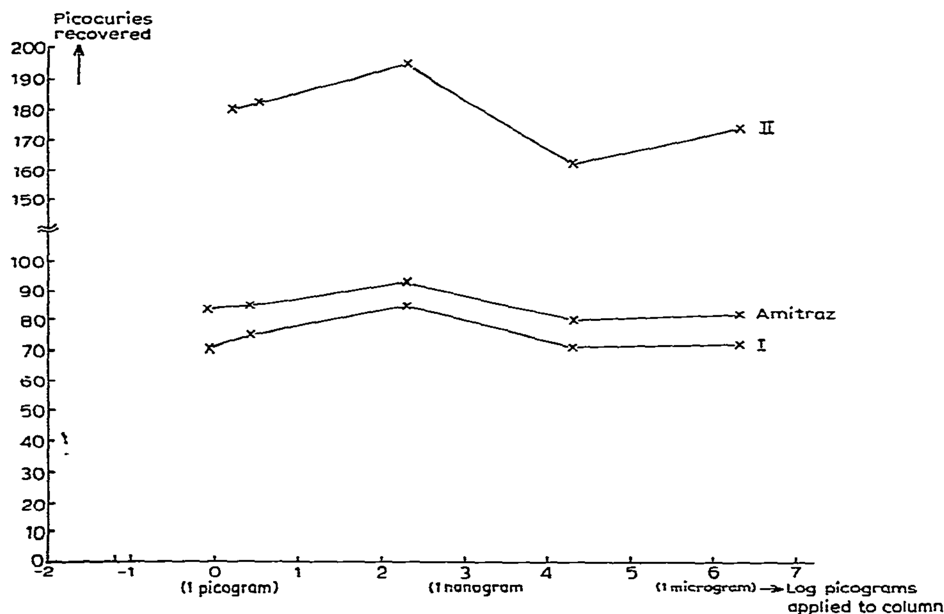


Fig. 4. Radioactivity of three compounds recovered at different carrier levels.

High-activity peaks have relatively little effect on other parts of the chromatogram as illustrated in Fig. 5. This mixture contained between 100 and 200 nCi per component and only a small increase in the radioactivity baseline was observed.

Contamination between runs is more difficult to control. Biological samples, particularly in the later stages of metabolism, can contain high proportions of polar metabolites, conjugates, etc. which will be strongly retained on the column. The pre-column serves to prevent these compounds from contaminating future runs. This was illustrated by following the injection of a biological extract containing a total activity of 157 nCi with an extract prepared from an untreated tick. The same pre-column was used for each injection and regenerated between injections as described above.

The sum of the activities found in the 40 fractions collected from the control sample was 190 pCi which represents a carry-over of about 1 part in $10^3$. No single bottle contained more than 30 pCi.

*Scintillation counting*

By selecting solvents for the LC separation which are low quenching, high counting efficiencies can be achieved. It was found that over the range of conditions employed in the present study, efficiencies varied in the range 50–54%. Thus, using plastic scintillation vials with backgrounds of 15–20 cpm and 10-min counts, activities of 20 pCi per vial could be counted with a coefficient of variability of 10% (calculated using the equation derived by Wyld[17]).

*Automation and data handling*

Using the Varian recycling solvent programmer and the automatic fraction

collection apparatus described above, manual interaction was reduced to a minimum. The output from the scintillation counter was fed off-line to a PDP 8F mini-computer where the activities in each vial were calculated by the external standard channels ratio method[18] and plotted as a histogram for each chromatography run. The computer output was printed on an ICL 7503 batch processing terminal. The fraction collector time interval was adjusted so that the index marks on the recorder trace aligned with the computer print-out. Thus the recorder trace, activity histogram and listing of activities were obtained on one sheet as shown in Fig. 5.

## DISCUSSION

Where the metabolic profile of a compound is known and the metabolites are available, small quantities of unlabelled compounds can be added to the extraction solvents to act was carriers. In this case chromatography is being carried out on microgram quantities of compounds which presents relatively few problems. The present study has shown that quantitative elution of the three compounds studied could be achieved down to the level of *ca.* 1 pg, and probably lower, without the addition of carriers. These compounds covered a range of polarities and all produced sharp symmetrical peaks. Where poorer peak shapes are produced, some losses on the columns might be expected.

The use of a detachable pre-column has been very effective in keeping the main column free of contamination; at least 10 consecutive runs could be performed without observing any increase in background counts even when tissue extracts containing up to 200 nCi were injected.

Much time could be saved by using a separate pre-column for each run and eluting these for acidic metabolites in batches of ten. The complete cycle time for the ether–hexane gradient, including changing of pre-columns and re-equilibration at the starting solvent composition was 45 min.

Good retention time reproducibility has been achieved: the ether–hexane mixture does not produce a significant pressure change during the gradient and so the retention time variability sometimes associated with syringe pumps[19] has not been observed.

Column life is excellent with the permanently bonded phase and no deterioration of the pre-columns has been observed through repeated cycles of acid- and base-treated solvents.

The technique is being applied to the measurements of metabolism and tissue distribution of amitraz and other chemicals in cattle ticks and the results of these studies will be reported elsewhere.

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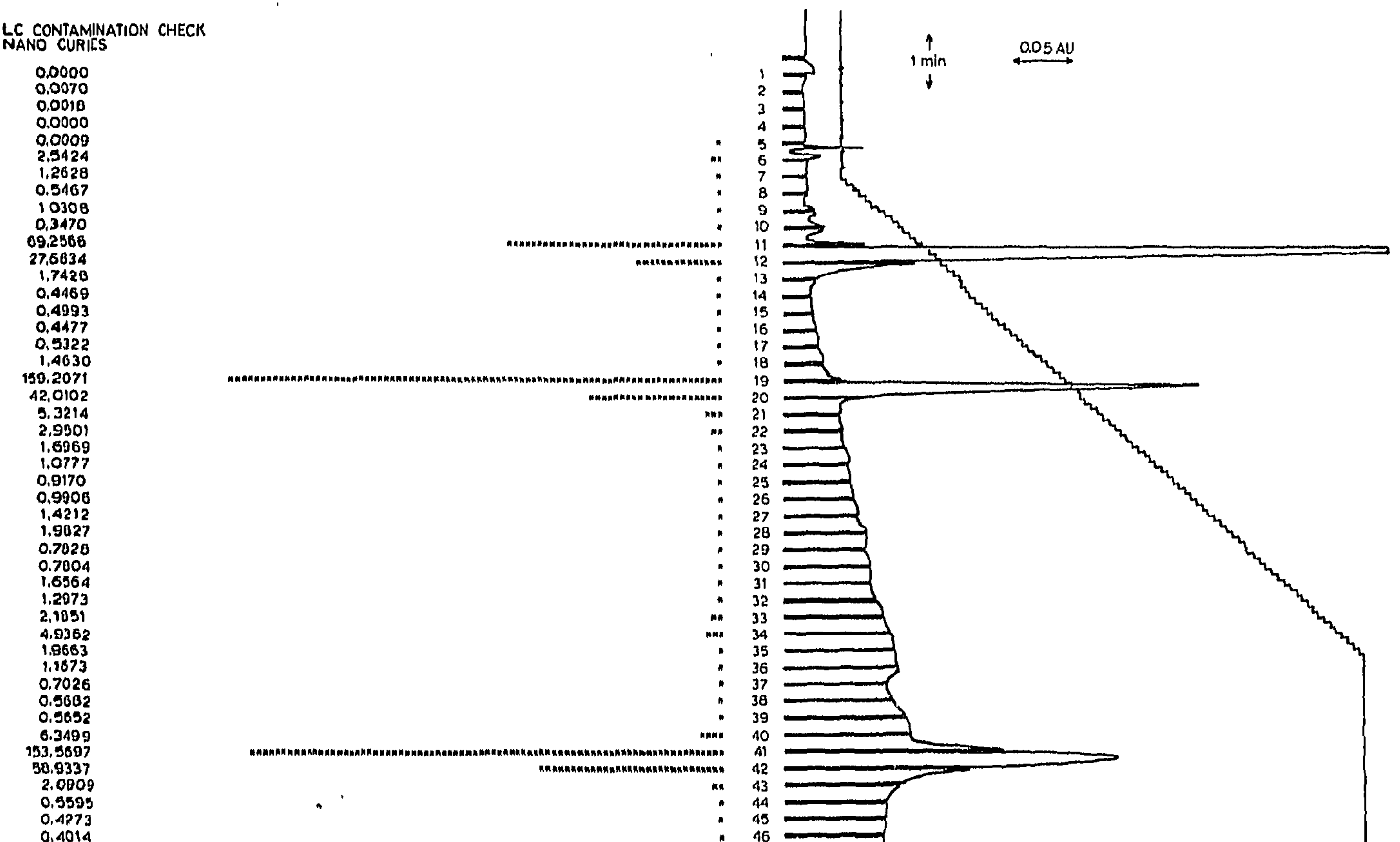


Fig. 5. Mass and radioactivity traces for [$^3$H]amitraz and compounds I and II; 100–200 nCi of each component injected. Conditions as in Fig. 3a.

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