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APPLICATION OF TWO-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN METABOLISM STUDIES OF IVERMECTIN

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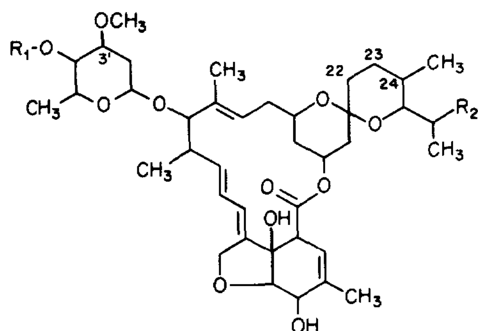
SUMMARY

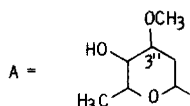
Two-dimensional high-performance liquid chromatography (HPLC) of alternate reversed-phase and normal-phase columns was used in the purification, quantification and identification of submicrogram quantities of drug residue and metabolites in tissues from animals dosed with ivermectin. In reverse isotope dilution assay of the parent drug, two-dimensional HPLC assured constant specific activity of the drug isolates. For identification of metabolites from liver tissue, HPLC in two dimensions not only facilitated the purification, but also provided information (capacity factor k') in both systems for the elimination of possible metabolite structures in the course of compound identification. These approaches exemplified by the ivermectin studies should be generally applicable in analyses of complex biological samples in which quantity is frequently a limiting factor.

INTRODUCTION

The practice of two-dimensional (or two-stage) high-performance liquid chromatography (HPLC) in various applications has been reported by many laboratories [1–3]. Two-dimensional HPLC analysis is defined as the use of two consecutive chromatographic steps for the purpose of separation, purification or analysis. The two dimensions are achieved either by one single mode of separation under two different conditions (e.g. mobile phases) [1] or by two different modes of separation, such as a cation-exchange mode followed by a reversed-phase mode [2,3]. The advantages of two-dimensional HPLC are obvious, as pointed out by other authors, when one visualizes a two-dimensional thin-layer chromatogram, which greatly facilitates the resolution of mixtures. In this paper we present a unique advantage of two-dimensional HPLC in the application to metabolite structure elucidation at submicrogram quantities, as exemplified by the metabolism studies on ivermection.



		R_1	R_2
I.	H_2B_{1a}	A	CH_2CH_3
II.	H_2B_{1b}	A	CH_3
III.	3''-O-Desmethyl-H_2B_{1a}	B	CH_2CH_3
IV.	3''-O-Desmethyl-H_2B_{1b}	B	CH_3
V.	H_2B_{1a}-Monosaccharide	H	CH_2CH_3
VI.	H_2B_{1b}-Monosaccharide	H	CH_3

A = 

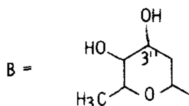
B = 

Fig. 1. Structures of ivermectin and its derivatives. Radioactive I and II used in metabolism studies were tritium-labeled at C-22 and C-23.

Ivermectin is a new broad-spectrum antiparasitic agent widely used in many food-producing animal species, such as cattle, sheep and swine [4–6]. It consists of two compounds, dihydroavermectin B_{1a} (H_2B_{1a}) and dihydroavermectin B_{1b} (H_2B_{1b}) (I and II in Fig. 1, respectively). The composition of ivermectin is such that H_2B_{1a} is $\geqslant 80\%$ and H_2B_{1b} is $\leqslant 20\%$. The application of two-dimensional HPLC in studies on ivermectin include quantitation of drug residue in animal tissues by reverse isotope dilution assay (RIDA) as well as metabolite isolation, purification and identification.

EXPERIMENTAL

Materials

Animal tissue samples from steers, swine and rats were obtained from experiments reported previously [7–9]. Animals were dosed either orally or subcutaneously at 0.3 mg/kg (steer, rat) or 0.4 mg/kg (swine) with ^3H-labeled ivermectin at a specific activity of at least 0.1 mCi/mg and radiopurity $>99\%$. Tissue samples were stored frozen at $-20°C$ until to be used. Unlabeled ivermectin was obtained from Chemical Data Department, Merck Sharp and Dohme Research Labs. The compound was $>97\%$ pure and consisted of H_2B_{1a} and H_2B_{1b} at a ratio

of 84.4 : 15.6. A standard solution of ivermectin at 20 μg/ml showed an absorbance of 0.730 O.D. at 245 nm, and calculated to *A*% of 365.

All solvents used in tissue fractionation and chromatography were HPLC grade from either Fisher Scientific (Fairlawn, NJ, U.S.A.) or J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was HPLC grade from Fisher Scientific.

High-performance liquid chromatography

Liquid chromatographs from Spectra Physics (SP 8700) and Lab Data Control (LDC Constametric) were used in all studies. Each liquid chromatograph was equipped with a Rheodyne Model 7125 sample valve with a syringe loading 200-μl sample loop and a Schoeffel Instrument Model 770 ultraviolet detector. A recorder–integrator (Hewlett Packard Model 3280) and a fraction collector (LKB Model 2111) were connected to the SP 8700 chromatograph. The LDC unit was equipped with a Chromatographic Control Module (CCM) and a fraction collector (LKB Model 2211). Fractions on HPLC charts were marked by an event marking device installed between the detector and the fraction collector. All chromatograms were monitored at 245 nm. A flow-rate of 1 ml/min was maintained in all analyses unless otherwise indicated.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

Columns used were Zorbax ODS (250 mm × 4.6 mm, DuPont, Wilmington, DE, U.S.A.) columns with CO: Pell ODS packed guard columns (Whatman, Clifton, NJ, U.S.A.). The mobile phase was acetonitrile–methanol–water (49.2 : 32.8 : 18, v/v) (condition A).

Normal-phase high-performance liquid chromatography (NP-HPLC)

Columns used were Zorbax Sil (250 mm × 4.6 mm, DuPont) columns with HC Pellosil packed guard columns (Whatman). Mobile phases were isooctane–ethanol at 90 : 10 (condition B) and 85 : 15 (condition C).

Reverse isotope dilution assay

The analysis of unchanged [^{3}H]ivermectin in animal tissues by RIDA was reported by our laboratories previously [9]. From the tissue extracts (e.g. liver, kidney, muscle, fat) added with ivermectin unlabeled ("cold") carrier before work-up, the two ivermectin components, H_2B_{1a} and H_2B_{1b}, were separated by RP-HPLC (condition A). The column effluents corresponding to each of these two compounds were combined, evaporated under nitrogen, and the specific activities determined in ethanol. The compounds were rechromatographed under NP-HPLC condition B or C and the specific activity of each compound was again determined. Ultraviolet spectra were recorded using a Perkin-Elmer Model 559 UV–VIS spectrophotometer. Radioactivity was assayed by scintillation counting in Instagel cocktail (Packard Instrument, Downers Grove, IL, U.S.A.) with a TriCarb Model 3310 spectrometer for at least 10 min. The external standard ratio method was used in all scintillation counting. Efficiency of 36–40% was generally obtained with tritium radioactivity.

Isolation of in vitro and in vivo metabolites from swine liver

Tritium-labeled ivermectin was incubated with pig liver microsomes in the presence of a NADPH-regenerating system, and metabolites were isolated and identified as previously reported [10]. In vivo metabolites in swine liver were isolated by solvent extraction of a 2-kg composite liver sample from seven and fourteen days post-dose swine followed by HPLC of the extract. Solvent extraction followed the published procedures in studies with steers, sheep and rats [7]. Chromatographic separation of the in vivo metabolites was by repetitive RP- and NP-HPLC, with retention times determined for all pure in vitro and in vivo metabolites under RP-HPLC condition A and NP-HPLC condition B.

Mix-sample chromatography

A small aliquot of the in vivo metabolite D-3 from swine liver (2000 dpm, ~9 ng) was chromatographed under NP-HPLC condition B. Another aliquot containing about an equal amount of radioactivity was mixed with 1–2 μg of the in vitro metabolite IV produced by swine liver microsomes [10] and chromatographed under the same condition. The column effluent under the UV absorbance peak of metabolite IV was recovered, evaporated to dryness under nitrogen and rechromatographed by RP-HPLC condition A. Aliquots of 0.2 ml from each column fraction were counted in Instagel to establish the radioactivity distribution profile.

RESULTS AND DISCUSSION

Purification of [^{3}H]ivermectin from tissues for HPLC–RIDA

Quantitation of the unchanged drug in animal tissues by RIDA was based on the difference between specific activity of the drug in the dose and that isolated from the tissue after dilution with a known amount of unlabeled carrier prior to the tissue extraction step [9,11,12].

In the conventional RIDA method, consecutive recrystallization is employed to achieve a constant specific activity in the analyte [11,12]. This can be difficult for drug residue analysis if the tissue residue levels are extremely low and the carrier dilution required for recrystallization is too great that accurate specific activity measurements are not possible. With the HPLC–RIDA method developed in our laboratories [9], less than 1 mg of unlabeled carrier was added and the isolated drug residue was repeatedly chromatographed until a constant specific activity was attained. Table I shows the results of specific activity measurements on H_2B_{1a} and H_2B_{1b} from steer and rat tissues after purifications by RP- and NP-HPLC. RP-HPLC was used in the first dimension from which H_2B_{1a} and H_2B_{1b} were separated and recovered according to the UV absorbance peaks of the cold carrier. After specific activity measurements, the drug isolates were chromatographed in the second dimension by NP-HPLC to confirm the specific activity. In one case (steer kidney) the second-dimension (NP-HPLC) purification significantly lowered the specific activity and accordingly the final percentage H_2B_{1a} in the tissue. A third chromatography again in the first dimension (RP-HPLC) then confirmed the specific activity. Purification achieved by the com-

TABLE I

PURIFICATION OF [^{3}H]IVERMECTIN H_2B_{1a} FROM ANIMAL TISSUES BY HPLC-RIDA

The amount of cold H_2B_{1a} carrier added in these assays was 791 μg

Tissue	HPLC No.	Mode	Specific activity (dpm/μg)	Radioactivity in drug (dpm)	Radioactivity in tissue sample (dpm)	H_2B_{1a} in tissue by RIDA (%)
Rat liver	1	RP	32.2	25470	31886	79.9
	2	NP	31.9	25233		79.1
	3	RP	31.8	25154		78.9
Steer kidney	1	RP	40.6	32115	36320	83.4
	2	NP	37.5	29663		81.7
	3	RP	36.9	29188		80.4
Rat kidney	1	RP	43.3	34250	41774	82.0
	2	NP	43.5	34409		82.4
Steer liver	1	RP	28.1	22227	30040	74.0
	2	NP	28.6	22623		75 3

bination of the two dimensions usually resulted in the lowering of the percentage drug calculated based on the specific activity and finally reached values that were within ±2%.

Identification of in vivo ivermectin metabolites

The tissue extract of a 2-kg composite swine liver sample contained about $8 \cdot 10^6$ dpm of tritium radioactivity. A profile of the radioactive components is shown in Fig. 2A by NP-HPLC under condition C. In this profile, the major group of metabolites eluted after the unchanged parent drug between 45 and 66 min, indicating higher polarity of the compounds in the mixture. When this group of radioactivity was recovered and rechromatographed by RP-HPLC condition A, it was resolved into at least four peaks (Fig. 2B) designated metabolites D-1, 2, 3 and 4, in order of increasing polarity. Metabolite D-3, when further chromatographed by NP-HPLC condition B, was purified from contamination by other metabolites, i.e. D-2 and D-4 (note that elution positions of D-2, D-3 and D-4 were reversed from before). D-3 was 62% in this sample and a total of ~5 μg purified D-3 was obtained after this procedure. A similar chromatographic procedure for D-2 afforded ~5 μg of the metabolite. The capacity factors (k') of these metabolites under both RP- and NP-HPLC conditions are listed in Table II. The k' values of the in vitro metabolites (by pig liver microsomes) are also shown in the same table for comparison. In the course of the in vivo metabolism studies of ivermectin, the list of in vitro metabolites served as a "library" of all potential metabolic transformation products. The problem then was to identify from the list those which might be the in vivo metabolites. By comparison of the k' values of the in vivo and in vitro metabolites from the first dimension (RP-HPLC), three compounds (a, c and d) from the in vitro source were similar to the in vivo metabolite D-2, and likewise two compounds (g and h) to metabolite

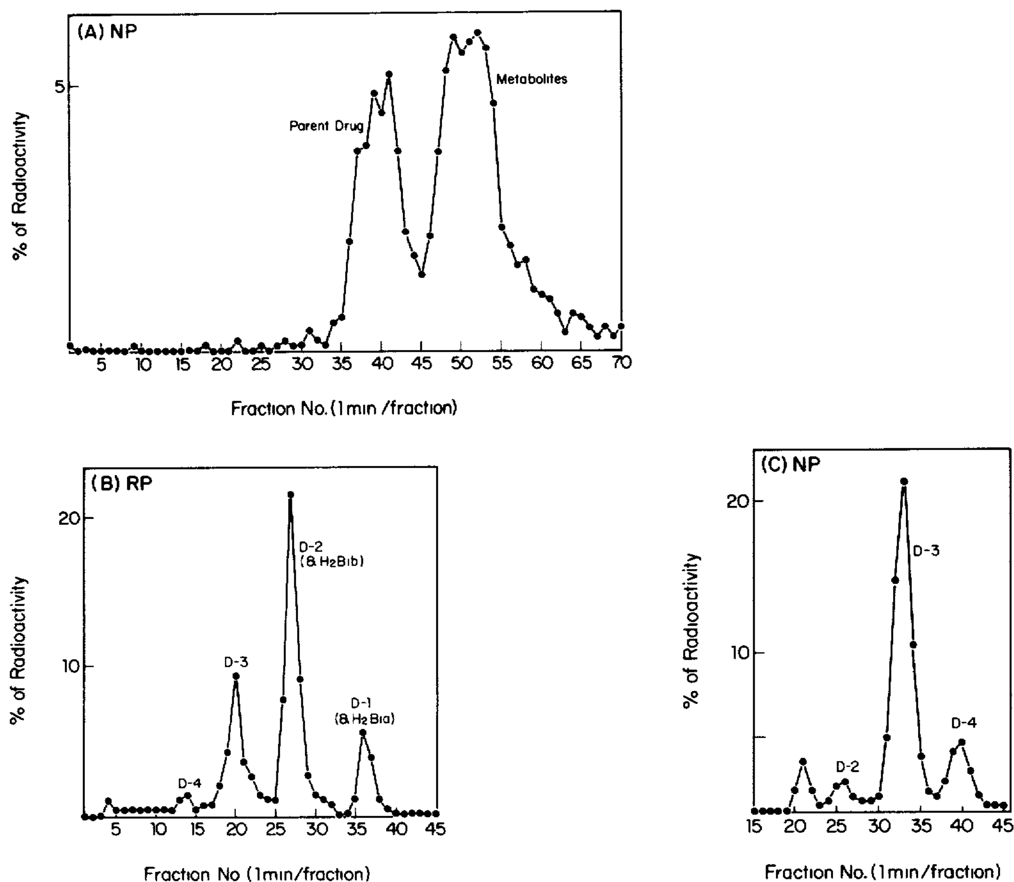


Fig. 2. Purification of polar swine liver metabolites by two-dimensional HPLC. (A) Final swine liver tissue extract, total radioactivity $1.5 \cdot 10^6$ dpm (10 μg drug equivalents) chromatographed by NP-HPLC condition C. Each column fraction was diluted to 4 ml with ethanol and 10 μl were withdrawn for scintillation counting. Fractions 45–70 were combined. (B) RP-HPLC of sample from (A) in condition A. Aliquot counted as in (A). Radioactivity pooled for D-3 and D-2 from fractions 16–24 and 25–42, respectively. (C) NP-HPLC of D-3 from (B) in condition B. D-3 was 62% in the sample.

D-3. However, with the added k' values from the second dimension (NP-HPLC), only one compound (i.e. c, or III in Fig. 1) from the in vitro collection chromatographed similarly with the in vivo metabolite D-2. Likewise, only one in vitro metabolite (h, or IV in Fig. 1) possessed k' values identical to D-3 in both dimensions.

The conclusion drawn from Table II eliminated the possibility of H_2B_{1a}-monosaccharide (H_2B_{1a}-MS, V in Fig. 1) being the structure of metabolite D-3. As a matter of fact, in the early stage of the study when only RP-HPLC was used for liver residue analysis, H_2B_{1a}-MS was thought to be the most probable structure for D-3 ($k' = 12$ for both compounds) until it was shown that the two compounds were widely separated by NP-HPLC, as seen from the k' values in Table II.

It is worth noting that despite the difference in k' for III and IV (likewise, D-2 and D-3) by RP-HPLC, they chromatographed similarly by NP-HPLC. This

TABLE II

RESOLUTION OF IVERMECTIN METABOLITES BY TWO-DIMENSIONAL HPLC

HPLC conditions: reversed phase (RP), condition A; normal phase (NP), condition B, as described in Experimental.

Compound	k'	
	RP	NP
H_2B_{1a} (I)	18	4
H_2B_{1b}	12	
H_2B_{1a}-MS (V)	9	6.3
H_2B_{1b}-MS (VI)	8	
In vivo metabolites		
D-2	12	10
D-3	9	10
In vitro metabolites		
From H_2B_{1a}-a	12	6.3
-b	—	8
-c (III)	12	10
-d	12	5
-e	11	6.7
From H_2B_{1b}-f	12	5
-g	9	7
-h (IV)	9	10

provided the essential clue that these metabolites were possibly analogues originated by similar metabolic conversion of H_2B_{1a} and H_2B_{1b}, which are analogues themselves. Other known analogues are the monosaccharides of the drug, H_2B_{1a}-MS (V) and H_2B_{1b}-MS (VI). Structures of III and IV were identified as 3″-O-desmethyl-H_2B_{1a} and 3″-O-desmethyl-H_2B_{1b}, respectively, from our previous studies [10].

The possible identity of metabolite D-3 with in vitro metabolite IV (3″-O-desmethyl-H_2B_{1b}) was substantiated by two-dimensional mix-sample chromatography as described in Experimental. With about 2000 dpm of D-3 (a mass of ~9 ng) and 1–2 μg of IV, the first-dimension HPLC (NP) recovered over 90% of the radioactivity under the UV absorbance peak of IV. Upon chromatography by the second dimension (RP), about 1200 dpm coeluted with IV. Using this method, the identity of metabolites D-2 with III was also confirmed. A final proof of identity in each case was arrived at by fluorescence derivatization [7,13,14] of the column eluate recovered after the second HPLC. As the product again showed coelution of the fluorescence peak and the radioactivity peak, the identities of these compounds based on results of two-dimensional chromatography were unequivocally established.

CONCLUSION

We have presented in this paper applications of two-dimensional HPLC in metabolism studies involving the assay of low-level residues of ivermectin and the isolation, purification as well as identification of its metabolites present in biological systems. On many occasions, the purity (or the identity) of a biological isolate can only be assured when it has been examined by at least two modes of separation. Two-dimensional chromatography is especially valuable when the analytes are at submicrogram levels and other analytical methods such as nuclear magnetic resonance, mass spectrometry and crystallization are not readily applicable. As shown in our study, documentation of HPLC retention properties of biological isolates in two dimensions greatly facilitates the elimination of unrelated compounds in the course of structure elucidation. This is especially crucial in the analyses of biological samples such as those from ivermectin studies in which the low tissue residue levels usually limit the recovery of metabolites to no more than a few micrograms even when a tissue sample of kilogram weight is used. The application of two-dimensional HPLC exemplified in these studies should be generally useful in analyses of complex biological mixtures.

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REFERENCES

1 G M Anderson, K.R. Schlicht and D J. Cohen, Anal. Chem., 55 (1983) 1399-1402.
2 N. Takahashi, Y. Takahashi and F.W. Putnam, J. Chromatogr., 266 (1983) 511-522.
3 F. Ernı and R.W. Frei, J. Chromatogr., 149 (1978) 561-569.
4 J.C. Chabala, H. Mrozik, R.L. Tolman, P. Eskola, A. Lusi, L.H. Peterson, M.F. Woods, M.H. Fisher, W.C Campbell, J.R. Egerton and D.A. Oslınd, J. Med. Chem , 23 (1980) 1134-1136
5 W.C. Campbell, M.H. Fisher, E.O Stapley, G. Albers-Schonberg and T.A. Jacob, Science, 221 (1983) 823-828.
6 R.W. Burg, B.M. Miller, E.E. Baker, J. Birnbaum, S.A. Currie, R. Hartman, Y.L. Kong, R.L. Monaghan, G. Olson, I. Putter, J.B. Tunac, H. Wallick, E.O. Stapley, R. Oiwa and S. Omura, Antimicrob. Agents Chemother., 15 (1979) 361-367.
7 S H.L. Chiu, E. Sestokas, R. Taub, R.P. Buhs, M. Green, R. Sestokas, W.J.A. VandenHeuvel, B.H. Arison and T.A. Jacob, Drug Metab. Dıspos., 14 (1986) 590-600.
8 S.H.L. Chiu, R. Taub, E. Sestokas, A Y H. Lu and T.A. Jacob, Drug Metab. Rev., 18 (1987) 289-302
9 S H.L. Chiu, R.P. Buhs, E. Sestokas, R. Taub and T.A. Jacob, J. Agrıc. Food Chem., 33 (1985) 99-102.
10 S H.L. Chiu, E. Sestokas, R. Taub, J. Smıth, B Arison and A.Y.H. Lu, Drug Metab. Dispos., 12 (1985) 464-469.
11 W.H. Graham and W E. Bornak, Anal. Chem., 45 (1973) 623-624.
12 J. Tolayessy, T. Braun and M. Kyrs, Isotope Dilution Analysis, Pergamon Press, Oxford, 1972, p. 23.
13 J.W. Tolan, P. Eskola, D.W. Fink, H. Mrozik and L.A. Zimmerman, J. Chromatogr., 190 (1980) 367-376.
14 P.C. Tway, J.C. Wood, Jr. and G.V. Downing, J. Agrıc. Food Chem., 29 (1981) 1059-1063.