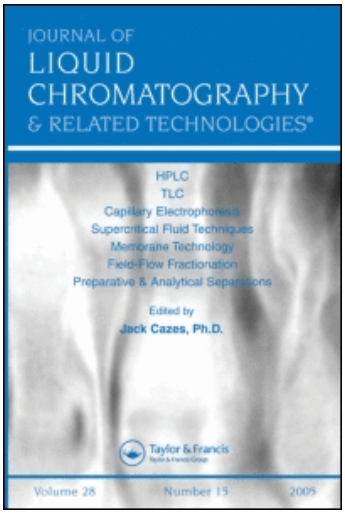

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HPLC ANALYSIS OF NOVEL ANTI-CANCER AGENTS-ILLUDINS AND THEIR ANALOGS

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ABSTRACT

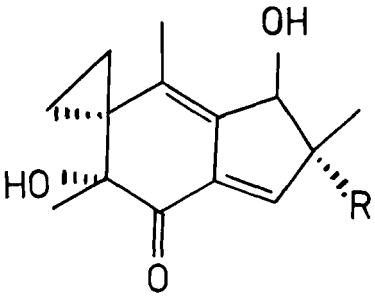
High performance liquid chromatography combined with liquid extraction and solid-phase extraction were used for determining the serum concentration of new anticancer drugs. Extraction recoveries were evaluated for wide range of concentrations in serum.

Adsorption and reversed-phase chromatographic modes were tested for the separation and drugs determination in model mixtures and actual clinical samples.

The HPLC assay developed was applied for farmacokinetic studies of acylfulvene in dog and rats.

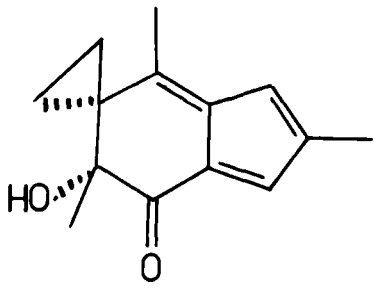
INTRODUCTION

Illudins and their analogs are novel natural low molecular products isolated from certain muschrooms /1,2/. They were previously evaluated in a variety of rodent tumor models and it was determined that some analogs were effective in vitro against human tumor cells and prefentially active against



Illudin S (R=CH_2OH)
Illudin M (R=CH_2)

Scheme 1.



Acylfulvene

Scheme 2.

leukemia /3-5/. They are selectively accummulated in certain of tumors including breast, colon and lung adenocarcinomas.

Recent experiments demonstrated that new analogs (acylfulvene and 6-OH methylacylfulvene) were very effective against primary metastatic breast carcinomas in dogs /6/. These metastatic tumors are not responsive to conventional anti - cancer agents.

In order to determine the reasons for the extreme cytotoxicity of illudins the detailed study of the structure and reactivity of these compounds and their derivates was published /7/.

Clarification of the mechanism of toxicity would enable to modify the structure so as to produce a compound with even greater selectivity in its toxicity towards leukemia cells than normal cells.

An X-ray crystalographic analysis of illudin S and illudin M were applied for this study.

The stability of illudins at low pH has also been examined /7/. When illudin M was dissolved in dilute HCl at room temperature it was converted to two chloroindantriols. The diastereoisomers were separated by chromatography. The structures were confirmed by NMR spectra. The reaction of illudin S with dilute

HCl is complicated by the presence of the primary hydroxyl. If an aqueous solution of illudin S is treated with dilute HCl, the precipitate has been identified as acylfulvene.

The histologic specifity of illudins S toxicity was studied and the Michelis constant for human myeloid leukemia HL 60 cells was determined /8/. The energy - dependent transport mechanism was detected in other mammalian tumor cells.

The aim of the presented work was to develop the HPLC method for the separation of illudins and their analogs and their determination in model mixtures and serum samples. As the preseparation steps both liquid and solid-phase extraction have been tested and results compared.

METHODS AND MATERIALS

Chemicals

Illudins S and M, acylfulvene and 6-OH methylacylfulvene were prepared at Department of Chemistry, University of California, San Diego, USA. They were dissolved in methanol before HPLC analysis in concentrations 100 μM as the stock solutions.

Methanol and acetonitril (HPLC grade, Fischer Scientific Co, Tustin, CA) were used for HPLC mobile phases. Ethylacetate, n-butylchloride (Burdick & Jackson Labs. Inc. Muskegon, MI) and methylene chloride (Fischer Scientific Co., Tustin, CA) were applied for liquid extraction procedures.

Instrumentation

A dual-pump high performance liquid chromatograph (Series 2) equipped with a variable wavelenght ultraviolet detector (Model LC-85 B), a recorder (Model PE-024) and 50 μl loop injector (all from Perkin Elmer Corp., Norwalk, CT) were used for all HPLC separations.

The columns C-8 and C-18, 5 μm particle size (4.6 mm diameter and 12.5 cm lenght; Perkin Elmer Corp.) were used.

Water Sep-Pak cartridges (C-18) were tested for solid-phase extraction of drugs from clinical samples.

UV-VIS spectrophotometer HP (Model 8450 A) was used for measuring UV spectra of all analyzed drugs.

Procedures

Liquid extraction : 1 ml of serum (with internal standard) was extracted with 4 ml of organic solvent (ethylacetate, n-butyl chloride and methylene chloride) shaking 3 minutes. After centrifuging (3000 x g for about 5 minutes), 2 ml of organic layer was evaporated by nitrogen to dryness and the rest was dissolved in 200/ul methanol (acetonitril) and 50/ul were injected into the chromatographic column.

Solid-phase extraction : Cartridge (C-18) was washed with methanol (2 ml), deionized water (2 ml). After loading 1 ml of serum (with internal standard) on the cartridge, it was washed with deionized water (2 ml) and the drug was eluated with methanol (acetonitril) (2 ml). The eluent was evaporated by nitrogen to dryness and the residue was dissolved in 200/ul of methanol (acetonitril).

RESULT AND DISCUSSION

Both adsorption and reversed-phase chromatography have been tested and reversed-phase column (C-8) was recommended for the HPLC study of illudins and acylfulvene. Mixtures methanol, acetonitrile and water were applied as mobile phases. For acylfulvene analysis (illudin M was used an internal standard (IS 1); it elutes with shorter elution time than analyzed acylfulvene. As the internal standard eluting with longer elution time 1-(2-thiazolylazo)-2-naphtol was applied (IS 2).

Before the optimization of HPLC conditions, UV spectra of analyzed compounds were measured and adsorption maxima evaluated :

acylfulvene : λ_1 = 237 nm; λ_2 = 326 nm
illudin M (IS 1) : λ_1 = 228 nm; λ_2 = 317 nm
IS 2 : λ = 224 nm

All HPLC analyses were realized using λ = 230 nm.

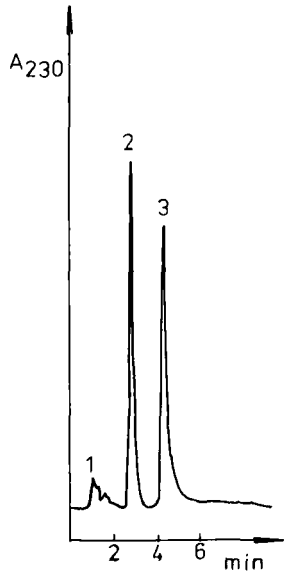


FIGURE 1. Chromatogram of illudin M (IS 1) and acylfulvene (standard mixture)
Column : C-8; 5 um
Mobile phase : 50 % acetonitril, 5 % methanol, 45 % water
Flow-rate : 1.3 ml/min, UV detection 230 nm
Peaks : 1 - solvent
2 - illudin M
3 - acylfulvene

For the succesful separation of acylfulvene and both internal standards (IS 1 and IS 2), the composition of the mobile phase was optimized and retention times, capacity ratios and chromatographic resolution values were evaluated. Both isocratic and gradient elutions have been studied but the isocrate mode is sufficient for the HPLC separation of all analyzed compounds. Retention times for acylfulvene and two internal standards for different composition of isocratic mobile phases are listed in Table 1.

For the analysis of clinical samples all three mobile phases are available as chromatographic resolutions (R_{ij}) for acylfulvene and the internal standard (IS 1 or IS 2) are

TABLE 1

Retention times for illudin M (IS 1), acylfulvene and IS 2 for isocratic mobile phases
Flow-rate : 1.3 ml/min

	Mobile phase	Illudin M (IS 1)	Acylfulvene	IS 2
1	55 % acetonitril 5 % methanol 40 % water	2.0 min	2.8 min	5.2 min
2	50 % acetonitril 5 % methanol 45 % water	2.8 min	4.4 min	6.8 min
3	45 % acetonitril 5 % methanol 50 % water	3.2 min	4.8 min	10.0 min

minimum R_{ij} = 1.25 what is sufficient for quantitative analysis.

For all recommended mobile phases the optimal flow-rates have been chosen. The optimization of flow-rates was rescricted by the demand of analysis time maximum 10 minutes.

The HPLC separation of acylfulvene and illudin M (internal standard) is demonstrated in Figure 1 and acylfulvene and IS 2 in Figure 2.

For the analysis of ethylacetate extracts of clinical samples the mobile phase 2 or 3 (Table 1) are recommended as they have enable the separation of the rest possible ethylacetate and illudin M as an internal standard for acylfulvene determination (Figure 3).

For clinical samples the liquid extractions with ethylacetate, n-butylchloride and methylene chloride were studied and extraction recoveries were compared for wide range of concentrations ($1/\mu$M - $100/\mu$M acylfulvene in serum; it means 0.22 - $22/\mu$g/ml). Extraction recoveries are shown in Table 2.

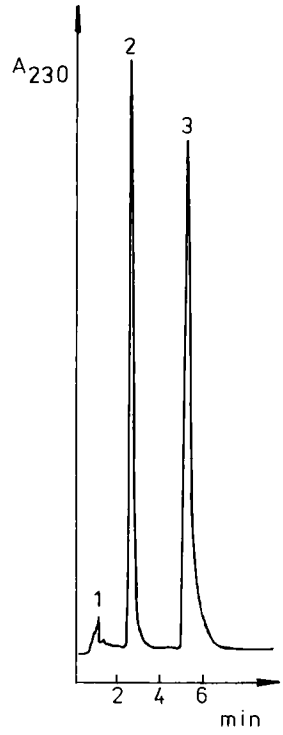


FIGURE 2. Chromatogram of acylfulvene and IS 2
(model mixture)
Column : C-8, 5/um
Mobile phase : 55 % acetonitril, 5 % methanol,
40 % water
Flow rate : 1.3 ml/min; UV detection 230 nm
Peaks : 1 - solvent; 2 - acylfulvene; 3 - IS 2

It is obvious from Table 2, that extraction recoveries are very hugh for ethylacetate. Extraction recovery for solid-phase extraction (10/uM of acylfulvene in serum) was 92.0 %.

A standard curve was prepared by adding acylfulvene to drug-free serum. The plot of concentration against peak-height ratio (drug and internal standard) was linear in the concentra-

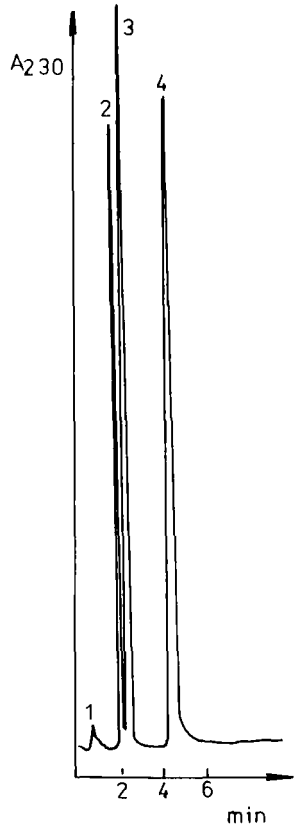


FIGURE 3. Chromatogram of etylacetate, illudin M and acylfulvene (model mixture)

Chromatographic conditions : as in Fig.1
Peaks : 1 - ethylacetate
2 - illudin M
3 - acylfulvene

tion range 0.2 - 50 ug/ml. The "least squares" regression equation was : y = 8.1 x - 1.78 (r = 0,9994). The detection limit of acylfulvene in serum was 75 ng/ml.

The analysis of 10 aliquots of drug-free serum supplemented with acylfulvene revealed the following coefficients of

TABLE 2

Extraction recoveries of acylfulvene from serum samples

Solvent	Concentration of acylfulvene (/uM)	Extraction recovery (%)	RSD (%) n=3
n-butyl chloride	100	68.2	4.2
	10	62.0	4.4
	1	52.6	5.1
methylene chloride	100	64.2	3.9
	10	61.1	4.2
	1	58.4	4.9
ethylacetate	100	93.6	5.2
	10	93.1	5.5
	1	88.7	6.4

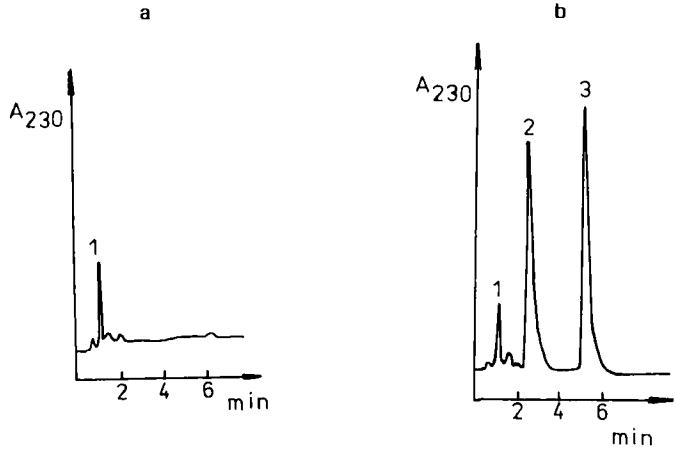


Fig.4a, b Chromatograms of serum samples
a) without acylfulvene
b) with acylfulvene (50/uM)
Conditions as in Fig.2
Peaks : 1 - solvent
2 - acylfulvene
3 - IS 2

variation (CVs) within run : 6.03 % and between run : 5.50% for the acylfulvene concentration 2.2/ug/ml (10/uM).

Chromatograms of serum samples without acylfulvene (blank) and serum spiked with 50/uM of acylfulvene are demonstrated in Fig.4a, b.

The HPLC assay developed was applied clinically for pharmacokinetic studies of acylfulvene in dog and rats. Concentrations about 5; 2.2 and 2/uM acylfulvene in dog serum samples were determined after 0.5 and 10 minutes from the infusion termination. The results dealing with pharmacokinetics in dog and rats have been discussed in detail in the paper of these authors submitted for Neoplasma 4, 1992.

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