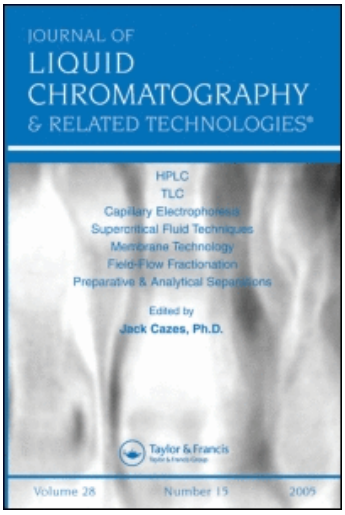

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Masuo Inoue[a]; Yoshiaki Kamano[a]; George R. Pettit[a]; Cecil R. Smith[a]
[a] Cancer Research Institute Arizona State University, Tempe, Arizona

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SEPARATION OF BUFOTALIN AND CINOBUFOTALIN BY PREPARATIVE LIQUID CHROMATOGRAPHY

Masuo Inoue, Yoshiaki Kamano, George R. Pettit*, and Cecil R. Smith

Cancer Research Institute
Arizona State University
Tempe, Arizona 85287

ABSTRACT

Complete resolution of bufadienolides by traditional column chromatography, or by thin-layer chromatography (TLC), is quite difficult. Separation of various bufadienolide conjugates by high performance liquid chromatography (HPLC) has been described, but not for resolution of the corresponding genins. A preparative HPLC procedure has been developed for resolving the difficultly separable bufotalin (1) and cinobufotalin (2). A Lobar B column packed with LiChromprep Si-60 was used to separate these bufadienolides employing a recycling procedure.

INTRODUCTION

Separation of typical bufadienolides by conventional column chromatography is difficult. When subjected to thin-layer chromatography (TLC), these compounds are also difficult to

*To whom correspondence should be addressed.

resolve.[1,2] Application of high-performance liquid chromatography (HPLC) to bufadienolide glycosides, and to other bufadienolide conjugates, has been described in several reports.[3-10] To our knowledge, the only two applications of HPLC or LC to separate the corresponding genins have been reported.[5,7] Neither addressed the particularly challenging resolution of bufadienolides (1) and (2). To meet our need for such a technique, we explored suitable conditions for preparative LC of bufadienolides (1) and (2) from the Japanese toad preparation Sen-So, which also contains a good number of related bufadienolides. Several solvent systems were examined by TLC, including the most promising systems reported by Komatsu and colleagues[1] and by Komatsu and Okano.[2]

EXPERIMENTAL

Instruments

The apparatus, arranged for low-pressure liquid chromatography at 50 psi, was comprised of: a FMI lab pump (Model RP-SYX), a FMI pulse dampener (Model PD-60-LF), four MTS sample injection slide valves (Pierce Chemical Co.) for sample injection and recycling, a Gilson fractionator and event marker (Model FC-220), and a Gilson UV-VIS Holochrome detector (Model HM) set at 254 nm.

Columns

For analytical separations, Lobar Size A columns (240 x 11 mm) packed with LiChroprep Si-60 (63-125 μm) were used.

Preparative separations were performed with Lobar Size B columns (310 x 25 mm) packed with LiChroprep Si-60 (63-125 μm) purchased from EM Reagents.

Sen-So Fractions

Trial separations were conducted with chromatographic fractions derived from the Japanese toad venom preparation Sen-So. The fractions had been previously evaluated by analytical TLC with the aid of authentic reference compounds, and contained mainly (1) and (2) with minor amounts of other bufadienolides.

Preparative Procedure

Preparative separations were begun in the normal mode. Samples were dissolved in the eluting solvent. Once the desired components were eluted into the column, the injection system was returned to the sample-loading position. After partially resolved bands passed through the ultraviolet detector, the operation was switched to the recycle mode if appropriate. Purity of eluted bands was judged by the degree of resolution of peaks recorded together with TLC results.

Thin-Layer Chromatography

Standard silica F-254 TLC plates, 0.25 mm (Brinkmann) were employed. Spots were visualized under ultraviolet light. Solvent systems examined are in Table I.

RESULTS AND DISCUSSION

A series of TLC solvent systems, investigated as an aid to HPLC resolution of bufotalin (1) and cinobufotalin (2), gave the

Table I

Efficacy of Various Solvent Systems in the TLC Separation of Bufotalin and Cinobufotalin.

	R_f Values		
	Bufotalin	Cinobufotalin	ΔR_f
hexane-ethyl acetate (1:5)	0.32	0.29	0.03
hexane-ethyl acetate-water (1:4:0.5)	0.30	0.27	0.03
hexane-ethyl acetate-methanol (1:3:0.5)	0.31	0.28	0.03
methylene chloride-methanol (95:5)	0.28	0.31	0.03
hexane-chloroform-acetone (4:3:3)	0.29	0.30	0.01

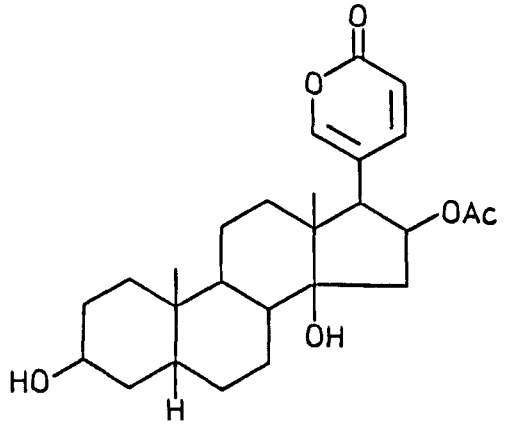
R_f values indicated in Table I. With a Lobar A analytical column, further exploration was pursued with solvent systems comprised of hexane-ethyl acetate-water and hexane-ethyl acetate-methanol. Additional optimization of the solvent system was achieved in preparative experiments summarized in Table II.

Experiments 1 and 2 (Table II) demonstrated that a single cycle with a Lobar B column did not adequately resolve bufotalin and cinobufotalin. In Experiment 3, a rather crude sample was applied -- one containing two unknowns in addition to the two bufadienolides of primary concern -- and the mixture was recycled three times. On the second recycle, the two unknown compounds began to overlap bufotalin and cinobufotalin. In Experiment 4, following removal of two unknown substances, satisfactory

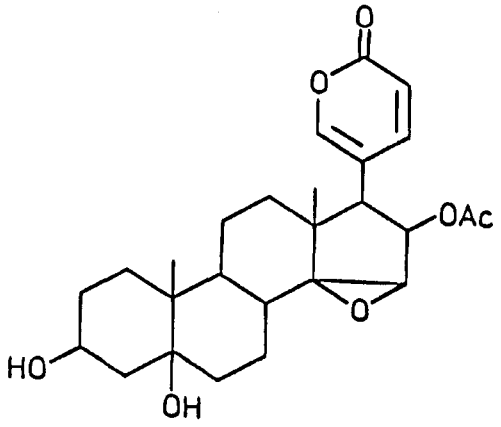
TABLE II.

Experimental Parameters in Resolution of Bufotalin and Cinobufotalin by HPLC with Lobar B Silica Gel Columns.

Experiment	Quantity Separation	Solvent	No. recycles	Flow Rate ml/min	Time	Comments
1		hexane-ethyl acetate-water (10:10:0.1)	0	2.3	26	Bufotalin band fairly pure but not cinobufotalin band.
2		hexane-ethyl acetate-water (10:10:0.2)	0	1.2	22	as above
3	1-10 mg	hexane-ethyl acetate-water (10:15:0.2)	3	3.5		Overlap of unknown compounds begins at second recycle.
4	1-10 mg	hexane-ethyl acetate-water (10:20:0.3)	3	6.4-7	10	Satisfactory separation with unknowns removed before recycling.
5	1.21 g	hexane-ethyl acetate-water (10:20:0.3)	3	5-6	12.5	99.4% recovery; bufotalin, 88.6% pure; cinobufotalin 91.7% pure.
6	59 mg	hexane-ethyl acetate-water (10:20:0.3)	4	7.5		29 mg bufotalin, 30 mg cinobufotalin recovered.
7	503 mg	hexane-ethyl acetate-water (10:20:0.3)	5	4.8-5.0	13	
8	781 mg	hexane-ethyl acetate-water (20:20:0.3)	2	3.8		99.2% recovered; 97.8% pure bufotalin, 98.3% pure cinobufotalin



(1) bufotalin



(2) cinobufotalin

resolution of bufotalin and cinobufotalin was achieved in three recycles. Subsequently, (in Experiment 5) a 1.21 g portion of the mixture was fairly well resolved with operating conditions similar to 4. Some degree of overloading was evident with this relatively large sample size; this might have been corrected by

using two columns in series. In Experiment 6, it was demonstrated that four recycles (same solvents as in 4 and 5) effected separation of a 59 mg sample of bufotalin and cinobufotalin to provide 97-98% purity of the two collected bands. Under conditions similar to experiment 6, 279 mg of the mixture yielded bufotalin and cinobufotalin with more than 98% purity (as judged by peak area obtained upon injection in an analytical column). However, this separation required five recycles. In the final experiment (No. 8), 628 mg of the mixture was resolved to provide each compound in a good state of purity by using two columns in series and only two recycles.

CONCLUSIONS

The preceding experiments demonstrate that bufotalin and cinobufotalin can be separated preparatively in a high state of purity by LC using Lobar B columns packed with LiChroprep Si-60 and the solvent system hexane-ethyl acetate-water (10:20:0.3), and with two to five recycles, depending on the sample load. For heavier loadings, use of two columns in series may be advantageous.

ACKNOWLEDGMENTS

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