Analysis of the Toad Poison Bufadienolides by Rod-Thin-Layer Chromatography with Flame Ionization Detector

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Summary

Analysis of the toad poison bufadienolides using the rod-thin-layer chromatography (Rod-TLC) with FID was undertaken. By the use of n-hexane/CH_2Cl_2/MeOH(11:8:1) as a developing solvent system, an effective separation of the eight bufadienolides - cinobufagin, resibufogenin, bufalin, bufotalin, desacetyl-cinobufagin, desacetyl-bufotalin, gamabufotalin and 3-oxo-cinobufagin - was obtained. Also, the reactivities of cinobufagin in oxidation and hydrolysis by using reaction mixture were checked by Rod-TLC. The contents of three major bufadienolides - resibufogenin, cinobufagin and bufalin - in the two kinds of Ch'an Su (the so-called "disk-like Ch'an Su" and "thin-plate Ch'an Su") were analyzed by the use of Rod-TLC (IATROSCAN).

1. Introduction

The toad poison bufadienolides has a novel steroidal A/B cis and C/D cis structure (Figure 1a) with an α-pyrone ring at C-17 position and exhibits a range of biological activites such as cardiotonic, blood pressure stimulating, respiration, antiviral and antineoplastic activities[1]. The Chinese traditional drug Ch'an Su, called "senso" in Japan, is a product of the skin secretions of the Chinese local toads, *Bufo bufo gargarizans* and related species, and is a good natural source of bufadienolides. For the determination of bufadienolides in Ch'an Su, the use of gas liquid chromatography (GLC)[2], high performance liquid chromatography (HPLC)[2][3] and thin-layer chromatography (TLC)[2][4][5] has been reported, but there have not been any reports on the utilization of Rod-TLC. Here, we wish to report on the analysis of bufadienolides in Ch'an Su by the useful Rod-TLC with flame ionization detector (FID) using IATROSCAN apparatus, in addition to the establishment of necessary basic techniques in separation and determination of bufadienolides. This method by Rod-TLC has been applied to the determination of lipids[6], the compounds in Chinese traditional drugs[7].

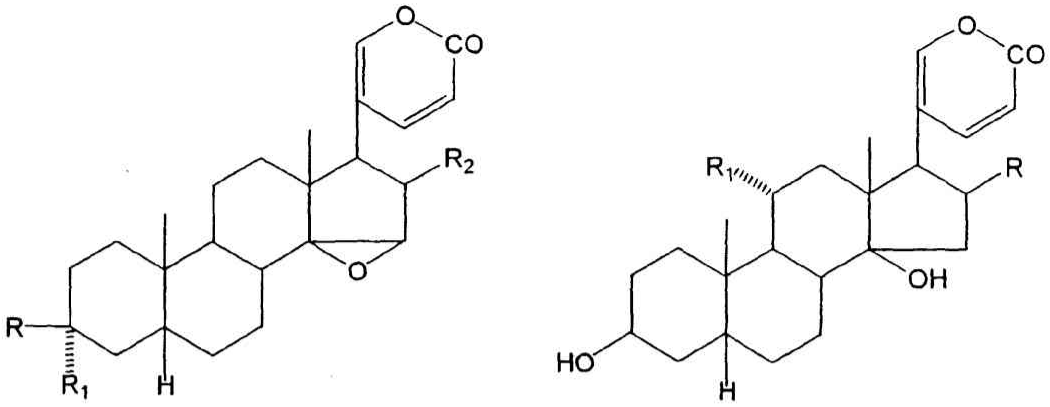
2. Experimental

2.1 Chemicals and Reagents

All solvents and reagents were purchased from commercial sources in the best available grade of purity.

2.2 Biological Material

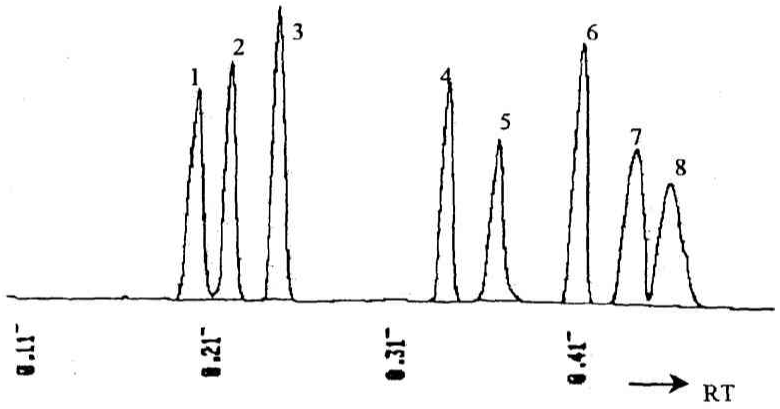
Two kinds of Ch'an Su (which were purchased in Hong Kong market) - disk-like Ch'an Su (CSD) and thin-plate Ch'an Su (CSP) - were used. The former, a brownish black disk of ϕ6cm, 0.8cm thick and 34.5g w.t., was made by Chinese Gifts Export Corp. (Qingdao). The other, a black rectangular thin-plate (15cm × 23cm × 0.1cm), was made by Shanghai Medicinal Herbs Import and Export Corp. (Shanghai). The bufadienolides (Figure 1a) - resibufogenin, cinobufagin, bufalin, bufotalin, desacetyl-cinobufagin, desacetyl-bufotalin and gamabufotalin - were isolated from Ch'an Su in our laboratory. 3-Oxo-cinobufagin was synthesized from cinobufagin by oxidation with CrO_3 in acetic acid[8].



resibufogenin R=OH, R_1=R_2=H
cinobufagin R=OH, R_1=H, R_2=$OCOCH_3$
desacetyl-cinobufagin R=R_2=OH, R_1=H
3-oxo-cinobufagin R_1+R_2=O, R_2=$OCOCH_3$

bufalin R=R_1=H
bufotalin R=$OCOCH_3$, R_1=H
desacetyl-bufotalin R=OH, R_1=H
gamabufotalin R=H, R_1=OH

(a) Structures of Bufadienolides



(b) Chromatogram of Bufadienolides by Rod-TLC

solvent - n-hexane/CH_2Cl_2/MeOH (11:8:1)
compounds (RT) - 1:3-oxo-cinobufagin (0.207), 2:cinobufagin (0.226), 3:resibufogenin (0.252), 4:bufalin (0.348), 5:bufotalin (0.372), 6:desacetyl-cinobufagin (0.419), 7:desacetyl-bufotalin (0.449), 8:gamabufotalin (0.468)

Figure 1

2.3 Instruments and Apparatus

For Rod-TLC experiment, the following apparatus were used. Main apparatus : IATROSCAN MK-5 TLC/FID analyser (IATROSCAN LABORATORIES, INC. Japan), Recorder : IATRO Recorder TC-11, Hydrogen generater for FID : GL Sciences HG 2500, Rod-TLC : CHROMATOROD-SIII (a silica gel rod, length : 13.3cm) (YATRON Co. Ltd.), Rod Dryer TK-8 (YATRON Co. Ltd.), Rod-TLC Chamber : DT 150 (YATRON Co. Ltd.). CHROMATOROD was stored in desicator and dried in Rod Dryer. The samples were plotted on CHROMATOROD by the use of HAMILTON micro syringe (1μL or 5μL).

2.4 Basic Condition of Rod-TLC

All procedures of Rod-TLC were performed by the guide book of IATROSCAN. The necessary basic condition was as follows. The temperature and humidity were 16℃(±2) and 50% (±5) respectively. Concentration of sample was set to 1-5mg/mL each and 1μL of sample solution was plotted on Chromatorod. For activation of Chromatorod, the blank scan was done three times. After activation, Chromatorod was stored at least for 5min in desicator. Scan speed was set at 30min/10cm. Sensitivity was set to 64. Developement Chamber was used in double cases by the utilization of preparative TLC chamber (20cm×20cm). Chromatorod was developed by each 10cm. After development, Chromatorod was dried at 67℃ in dryer.

2.5 Developing Solvents

First, selected good solvents for silica gel TLC such as n-hexane/$CHCl_3$/acetone (4:3:3) or n-hexane/ethyl acetate (6:4) were tested[5]. However, not all solvents were good for Rod-TLC separation. After an examination of some other solvents, n-hexane/CH_2Cl_2/MeOH system was found to be good as developing solvent for Rod-TLC. For separation of non-polar bufadienolides such as resibufogenin and cinobufagin, especially the use of the 11:8:1 ratio of n-hexane/CH_2Cl_2/MeOH was best. As occasion demands, the ratio of solvent could be changed.

2.6 Extraction of Ch'an Su

The 10g of powdered Ch'an Su was extracted three times with 30mL of $CHCl_3$ for each

8hr at room temperature under stirring. Additionally, third extraction was extended for 10 min under condition of refluxing. All $CHCl_3$ solution, after filteration, were combined and evaporated to dryness. The yields indicated in Table 1.

Table 1 Determination of Amounts of The $CHCl_3$ Extracts and Bufadienolides in Ch'an Su

Ch'an Su		$CHCl_3$ extracts	resibufogenin (%)	cinobufagin (%)	bufalin (%)
disk-like[a]	10g	2.8g (28%)	3.0 (2.46[2], 3.4[5])	4.7 (5.22[2], 5.0[5])	1.8 (2.05[2], 1.8[5])
thin-plate[b]	10g	2.3g (23%)	2.8 (3.27[2], 5.6[5])	5.7 (5.80[2], 5.6[5])	2.3 (2.37[2], 2.4[5])

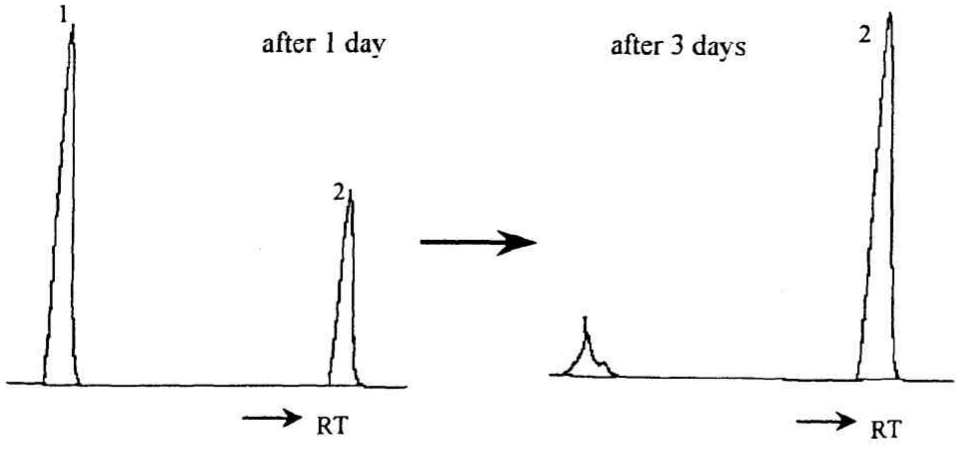
a : a brownish black disk of ϕ6cm, 0.8cm thick and 34.5g w.t.
b : a black rectangular thin-plate (15cm×23cm×0.1cm)
2) and 5) were same as the references in the text

2.7 Preparation of Calibration Curves

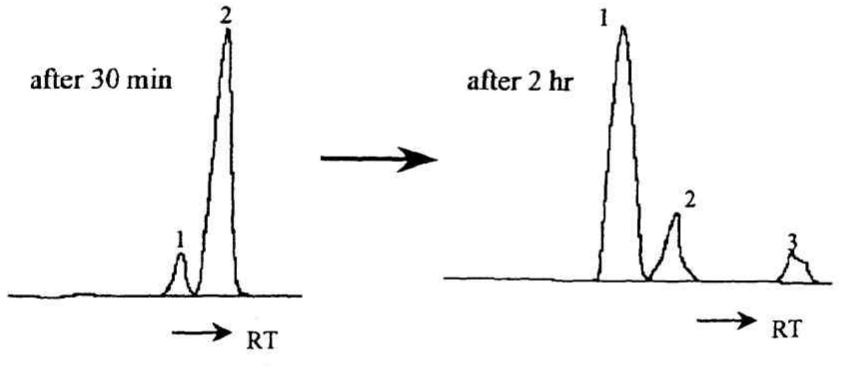
Each pure authentic sample of cinobufagin, resibufogenin and bufalin in $CHCl_3$/MeOH (9:1) was plotted with the ratio of 0.2-1μg by a micro syringe on each of 10 Chromatorod. After dryness, each Chromatorod was developed in solvent. The dried Chromatorod was scanned by IATROSCAN apparatus. For preparation of calibration curves, each avarage of 10 scan by machine was used.

2.8 Oxidation and Hydrolysis of Cinobufagin

(1)To a solution of cinobufagin (2mg) in MeOH (1mL)-H_2O (0.2mL), the ionexchange resin CG-400 (OH^-form) (12mg) was added. The mixture was stirred at room temperature for 72hr.
(2)To a solution of cinobufagin (2mg) in acetic acid (0.5mL), CrO_3 (1.1mg) was added. The reaction was conducted at 15-18℃ for 3hr. Formation of desacetyl-cinobufagin and 3-oxo-cinobufagin were checked by Rod-TLC[8].



(a) Chromatogram on Hydrolysis of Cinobufagin
solvent - n-hexane/CH_2Cl_2/MeOH (9:7:1)
compounds (RT) - 1:cinobufagin (0.22), 2:desacetyl-cinobufagin (0.39)



(b) Chromatogram on Oxidation of Cinobufagin
solvent - n-hexane/CH_2Cl_2/MeOH (10:8:1)
compounds (RT) - 1:3-oxo-cinobufagin (0.19), 2:cinobufagin (0.23),
3:by-product (0.29)

Figure 2 Chromatogram of The Reaction Mixture of Cinobufagin

3. Results and Discussion

3.1 Separation of Bufadienolides by Rod-TLC

For separation of bufadienolides, the use of solvent n-hexane/CH_2Cl_2/MeOH (11:8:1) was effective. Figure 1b showed a whole separation of natural bufadienolides - resibufogenin, cinobufagin, bufalin, bufotalin, desacetyl-cinobufagin, desacetyl-bufotalin and gamabufotalin - in addition to a synthetic 3-oxo-cinobufagin. Separation of cinobufagin (peak 2), resibufogenin (peak 3) and bufalin (peak 4) was excellent. The result led us to an analysis of these major bufadienolides in Ch'an Su. Also, separation of cinobufagin and desacetyl-cinobufagin (peak 6) was very good.

3.2 Application of Rod-TLC to Examination of The Reactivities of Cinobufagin

The reactivities of cinobufagin in oxidation and hydrolysis by Rod-TLC were examined. In both cases, the reaction mixture was plotted directly on Chromatorod. The ratio of products was determined by the concentration (%) of IATROSCAN. Figure 2a showed the formation of desacetyl-cinobufagin (peak 2) from cinobufagin (peak 1) by hydrolysis with the ionexchange resin CG-400 (OH^-form). After 3 days the 90% formation of desacetyl-cinobufagin was observed. On the other hand, Figure 2b showed the formation of 3-oxo-cinobufagin (peak 1) from cinobufagin (peak 2) by CrO_3 oxidation in acetic acid. It was found that the reaction 80% after 2hr from the starting and a new by-product was formed.

3.3 Analysis of Resibufogenin, Cinobufagin and Bufalin in Ch'an Su

The calibration curve (Figure 3) was constructed by plotting the ratio of the peak area ($\mu V \cdot sec$) of each bufadienolides with IATROSCAN, whereby satisfactory linearity was observed. By this calibration curve, three major bufadienolides in Ch'an Su were determined. In Figure 4, chromatograms of both CSD and CSP were indicated together with the estimation of retention time (RT), peak area ($\mu V \cdot sec$) and concentration (%) by IATROSCAN. The yields of bufadienolides were indicated in Table 1, after adjustment to the extracts of Ch'an Su. The results were similar to the data obtained by the color densitometric determination with Al_2O_3 plate[2] and by the UV two waves SCANNER densitometric determination with SiO_2 plate[5] respectively. Although the yields of both cinobufagin in CSD and resibufogenin in CSP were slightly smaller than those of the references, the small slight difference could be conpensated by the difference of each Ch'an Su.

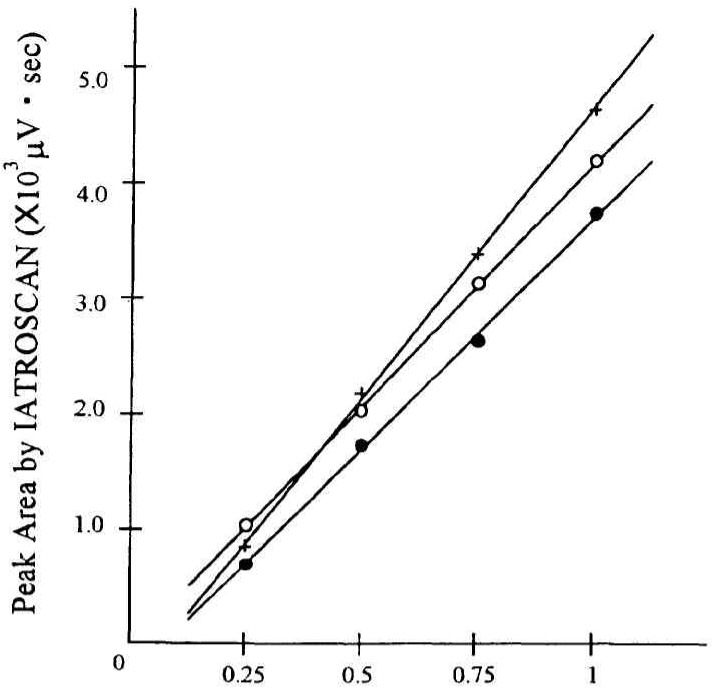


Figure 3 Calibration Curves of Bufadienolides by Rod-TLC

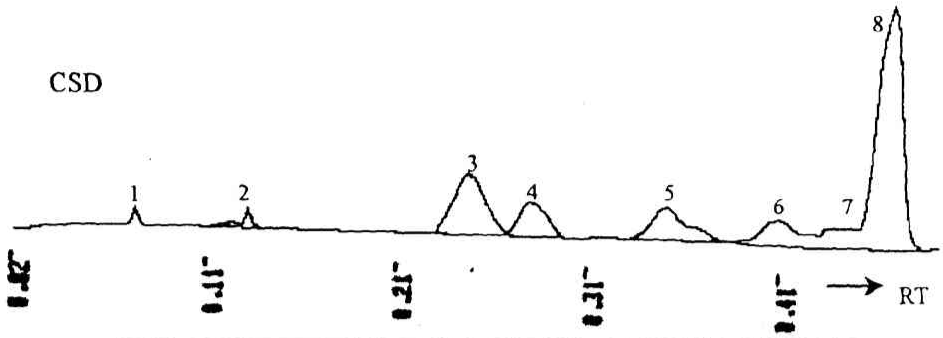
+—+ bufalin y=(5X10^6)x-359.54

o—o resibufogenin y=(4X10^6)x-192.83

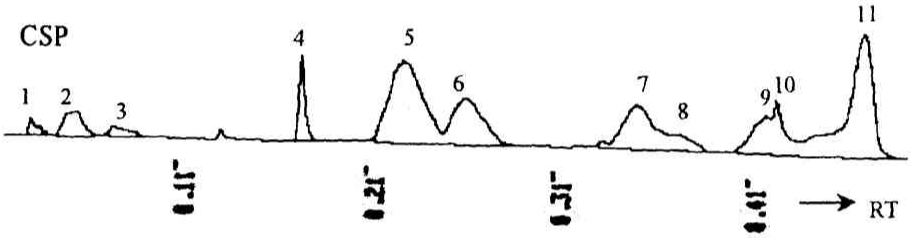
●—● cinobufagin y=(4X10^6)x-283.42

4. Conclusion

Separation of bufadienolides by Rod-TLC was very effective and useful. Some good solvents for development were found, analysis of other bufadienolides could be completed. Determination of reactivities of cinobufagin by oxidation and hydrolysis was a success for the use of Rod-TLC. Also, it was fruitful that the analysis of resibufogenin, cinobufagin and bufalin in Ch'an Su was established by Rod-TLC.



No.	compounds	RT	peak area(μV · sec)	concentration(%)
1		0.130	247	1.4132
2		0.140	242	1.382
3	cinobufagin	0.255	2935	16.7408
4	resibufogenin	0.275	1874	10.6912
5	bufalin	0.351	1110	6.3307
6		0.412	1138	6.4952
7		0.436	402	2.2947
8		0.468	9582	54.6517
total			17530	100.0000



No.	compounds	RT	peak area(μV · sec)	concentration(%)
1		0.046	281	1.4461
2		0.069	806	4.1458
3		0.087	454	2.3381
4		0.182	919	4.7231
5	cinobufagin	0.238	4773	24.5180
6	resibufogenin	0.261	2377	12.2122
7	bufalin	0.355	1926	9.8915
8		0.378	623	3.2044
9		0.421	1229	6.3122
10		0.427	1050	5.3954
11		0.469	5025	25.8126
total			19463	100.0000

Figure 4 Rod TL Chromatogram of The Ch'an Su Extracts and Analysis of Bufadienolides by IATROSCAN

solvent - n-hexane/CH_2Cl_2/MeOH (11:8:1)
sample - CSD ($CHCl_3$ extract of disk-like Ch'an Su)
CSP ($CHCl_3$ extract of thin-plate Ch'an Su)

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