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Note

Studies on steroids

CXIV. Separation of cardiotonic steroid conjugates by high-performance liquid chromatography

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The isolation and characterization of cardiotonic steroid conjugates from the Japanese toad has been carried out previously in these laboratories[1–9]. More detailed studies were required in order to establish a method for the separation of steroid conjugates in the toad venom. In recent years, considerable attention has been focused to the application of high-performance liquid chromatography (HPLC) to the separation and purification of physiologically active natural products[10]. This paper describes the separation of cardiotonic steroid conjugates by HPLC and the relationship between the structures and the retention values of homologous compounds.

EXPERIMENTAL

Instruments

The apparatus used was a Waters Model ALC/GPC 202 R401 high-performance liquid chromatograph equipped with an ultraviolet detector monitoring the absorbance at 254 and 280 nm. A μBondapak C_{18} column ($^1/_4$ in. × 1 ft.) (Waters Assoc., Milford, Mass., U.S.A.) was used under ambient conditions. The test samples were applied to the chromatograph by a Waters Models U6K sample loop injector with an effective volume of 2 ml.

Materials

Pregnadienolone (3β-hydroxy-5,16-pregnadien-20-one) 3-suberoylarginine ester and its homologues were synthesized according to the *p*-nitrophenyl ester method[11,12] in these laboratories. Bufalin 3-hemisuccinate, 3-hemiglutarate, 3-hemiadipate and 3-hemisuberate and cinobufagin 3-hemisuberate were kindly supplied by Dr. Y. Kamano, and bufalin 3-hemipimelate was prepared in the usual manner in these laboratories. Other bufogenin 3-dicarboxylic acid half-esters, bufogenin 3-sulphates, bufotoxins and their homologues were obtained from the skin of the Japanese toad[1–9].

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Preparation of toad venom sample

The fresh venom secreted from the parotid glands of the Japanese toad was immediately diluted with chloroform–methanol (1:1), concentrated *in vacuo* and then submitted to partition chromatography on silica gel employing chloroform–methanol–water (80:20:2.5) as a solvent[1,2,9]. Bufotoxins were thus divided into three fractions (Fractions I, II and III).

RESULTS AND DISCUSSION

Initial work was directed towards the separation of a variety of bufotoxins, namely bufogenin 3-suberoylarginine esters. As shown in Fig. 1a, these substances were well separated into five peaks, but difficulties were encountered in the differentiation of cinobufotalitoxin and marinobufotoxin from arenobufotoxin and resibufotoxin from cinobufotoxin when the methanol–water system was employed. These two groups, however, could be resolved into the individual components by the use of tetrahydrofuran–water as eluent (Fig. 1b and 1c). It is of interest that the

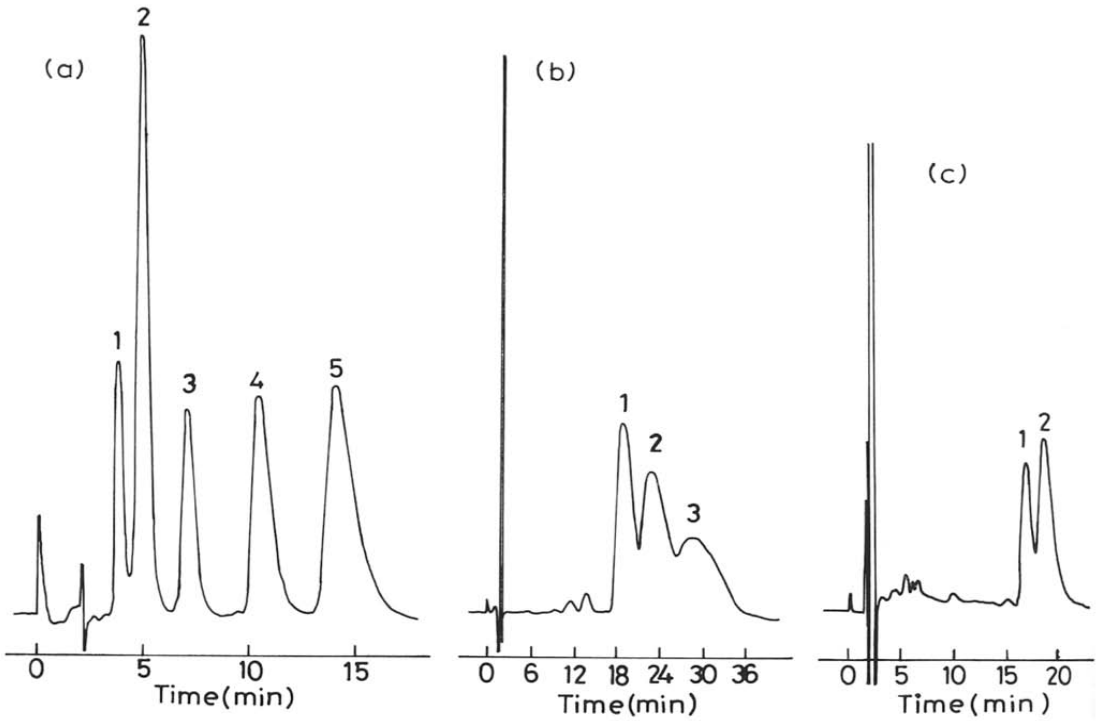

Fig. 1. (a) Separation of a mixture of bufotoxins. 1 = Gamabufotalitoxin; 2 = arenobufotoxin, cinobufotalitoxin, marinobufotoxin; 3 = vulgarobufotoxin; 4 = bufalitoxin; 5 = cinobufotoxin, resibufotoxin. Conditions: μBondapak C_{18} column; eluent, methanol–water (2:1), 1.5 ml/min; detection, 280 nm. (b) Separation of marinobufotoxin (1), cinobufotalitoxin (2) and arenobufotoxin (3). Eluent: tetrahydrofuran–water (1:3), 2 ml/min. Other conditions as in (a). (c) Separation of resibufotoxin (1) and cinobufotoxin (2). Eluent: tetrahydrofuran–water (1:2), 1.5 ml/min. Other conditions as in (a).

order of elution of these bufotoxins was nearly identical with that of corresponding genins under the same conditions[13].

Gamabufotalitoxin and its homologues (gamabufotalin 3-pimeloyl-, 3-adipoyl- and 3-succinoylarginine esters) were completely separated, as illustrated in Fig. 2. A linear correlation between the logarithm of the retention time relative to each genin and the number of methylene in the dicarboxylic acid moiety was observed for gamabufotalitoxin homologues, bufalitoxin homologues, cinobufotoxin homologues and pregnadienolone 3-suberoylarginine ester homologues (Fig. 3).

On the basis of these results, the characterization of cardiotonic steroid conjugates in the fresh venom secreted from the parotid glands of Japanese toad (*Bufo vulgaris formosus* Boulenger) was then undertaken. The specimen was fractionated into three portions by partition chromatography on silica gel and each fraction was then submitted to HPLC. The presence of cinobufagin 3-succinoylarginine ester and vulgarobufotoxin (Fraction I); arenobufotoxin, resibufogenin 3-succinoylarginine ester, bufalin 3-adipoylarginine ester, bufalin 3-pimeloylarginine ester and bufalitoxin (Fraction II); and gamabufotalin 3-succinoylarginine ester, gamabufotalin 3-adipoylarginine ester, gamabufotalin 3-pimeloylarginine ester, gamabufotalitoxin and bufalin 3-succinoylarginine ester (Fraction III) in these three fractions was demonstrated (Fig. 4). Closely related compounds that possess the same dicarboxylic acid residue and differ in the genin moiety, for instance 3-adipoylarginine esters of cinobufagin and resibufogenin, could not be separated when aqueous methanol was used as an eluent. However, these mixtures were resolved with success by the use of tetrahydro-

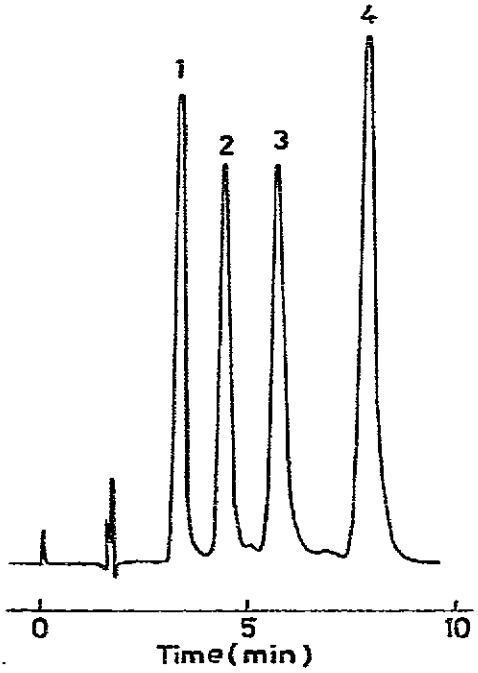

Fig. 2. Separation of gamabufotalitoxin (4) and its homologues. 1 = Gamabufotalin 3-succinoylarginine ester; 2 = gamabufotalin 3-adipoylarginine ester; 3 = gamabufotalin 3-pimeloylarginine ester. Eluent: methanol–water (5:4), 1.5 ml/min. Other conditions as in Fig. 1a.

furan–water as eluent. It is evident from the results that the toad venom contains at least 20 kinds of bufotoxin homologues.

Subsequent efforts were focused on the separation of bufogenin 3-dicarboxylic acid half-esters. As illustrated in Fig. 5a, the addition of ammonium dihydrogen orthophosphate to the solvent was found to be effective for the separation of these half-esters, with the exception of desacetylcinobufagin 3-hemisuccinate, sarmentogenin 3-hemisuberate and gamabufotalin 3-hemisuberate. This mixture, however, could be readily resolved into three components when the tetrahydrofuran–phosphate

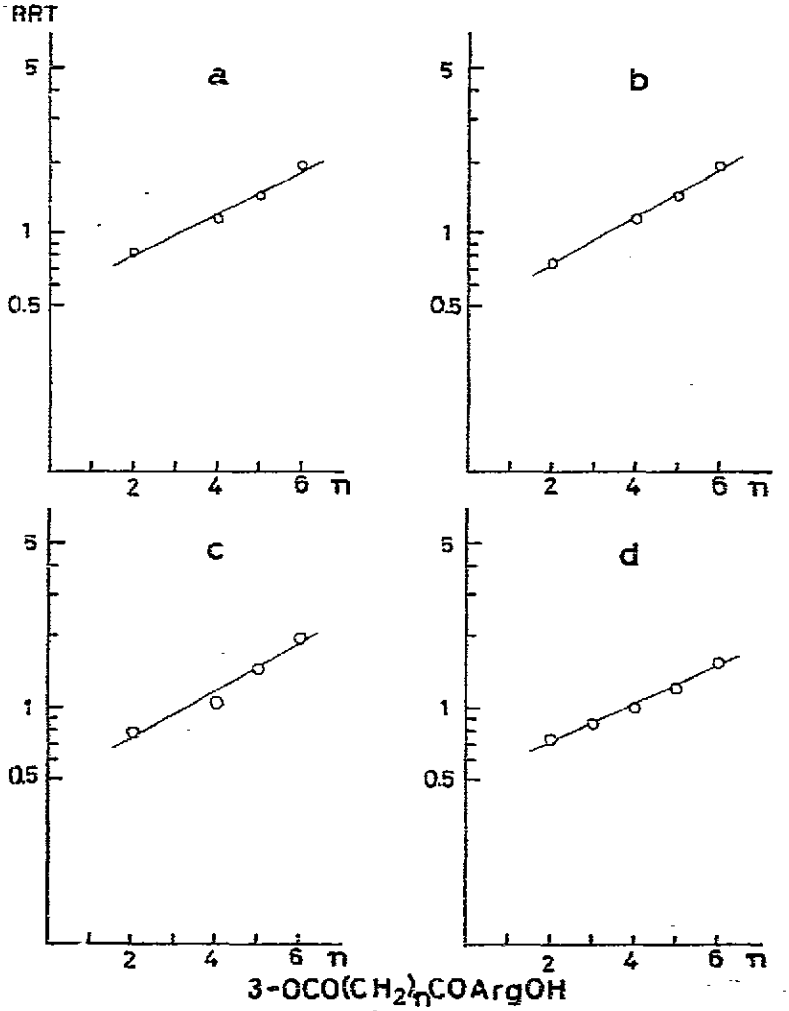

Fig. 3. Relationship between the relative retention time and the number of methylene groups in the dicarboxylic acid moiety. Conditions: μBondapak C_{18} column; eluent, (a) methanol–water (2:1), 1.5 ml/min, (b) methanol–water (2:1), 2 ml/min, (c) methanol–water (5:4), 1.5 ml/min, (d) methanol–0.02 *M* $NH_4H_2PO_4$ (3:1), 1.5 ml/min; detection, (a) 280 nm, (b) 280 nm, (c) 280 nm, (d) 254 nm. Retention time of internal standard: (a) bufalin, 6.2 min; (b) cinobufagin, 6.1 min; (c) gamabufotalin 3.8 min; (d) pregnadienolone, 6.9 min.

system was substituted for the methanol–phosphate system (Fig. 5b). A homologous mixture of bufalin 3-hemisuberate, 3-hemipimelate, 3-hemiadipate, 3-hemiglutarate and 3-hemisuccinate could be well separated by the use of aqueous methanol containing ammonium dihydrogen orthophosphate (Fig. 6). As shown in Fig. 7, a linear relationship was observed between the logarithm of the retention time relative to the

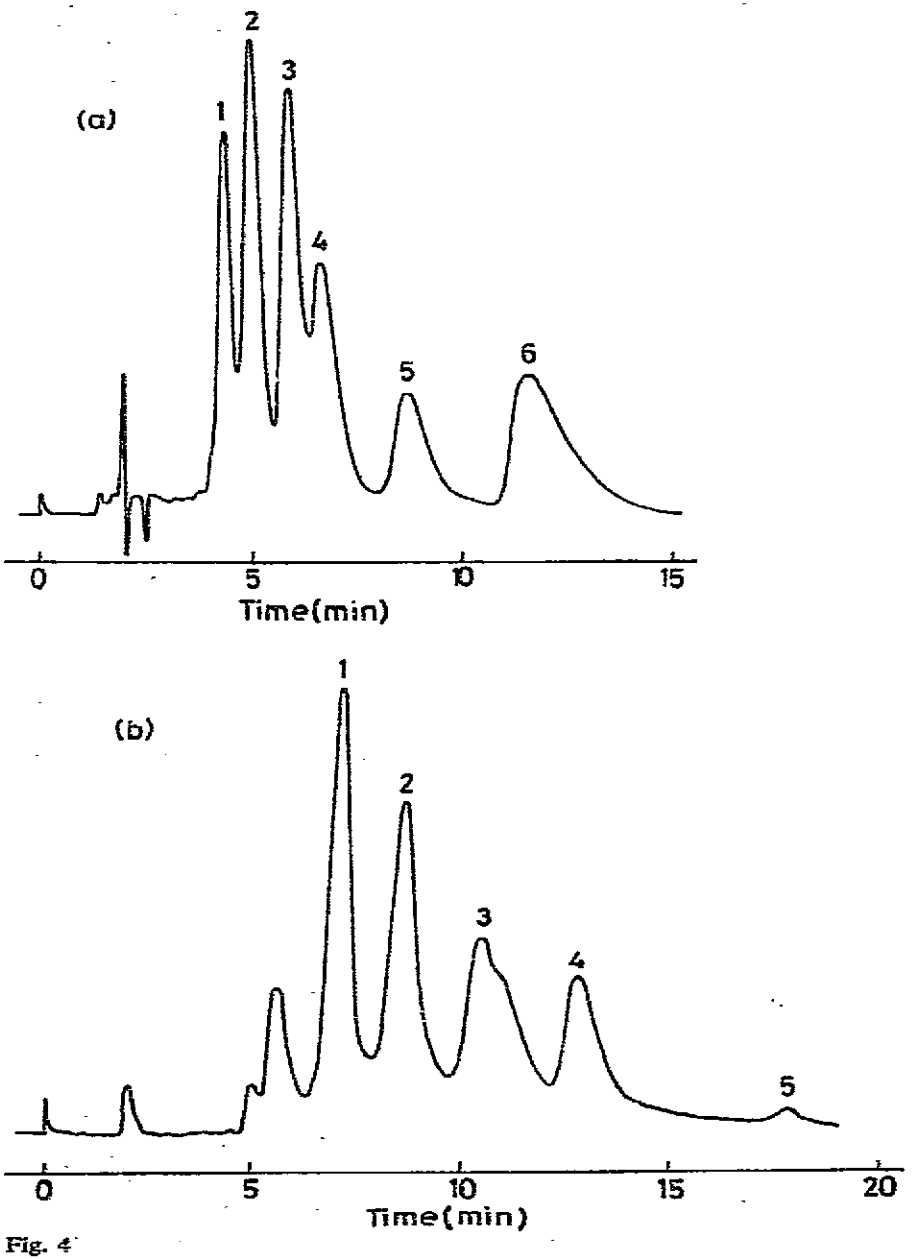

Fig. 4

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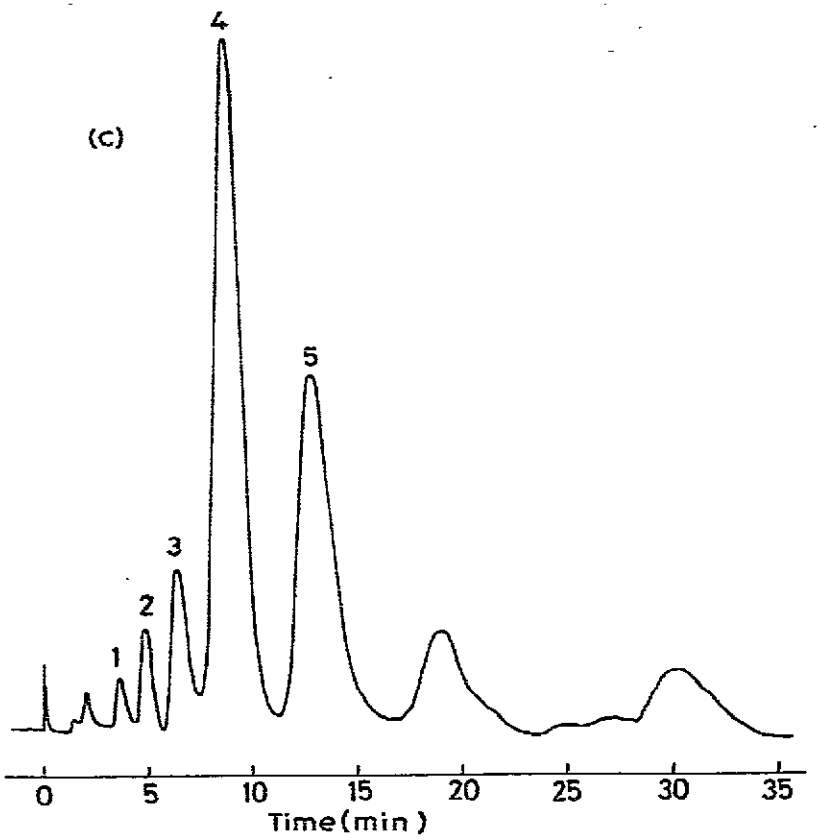

Fig. 4. Chromatograms of bufotoxin homologues in the venom of Japanese toad. (a) Fraction I: 1 = cinobufotalitoxin, marinobufotoxin; 2 = cinobufagin 3-succinoylarginine ester; 3 = vulgarobufotoxin; 4 = cinobufagin 3-adipoylarginine ester, resibufogenin 3-adipoylarginine ester; 5 = cinobufagin 3-pimeloylarginine ester, resibufogenin 3-pimeloylarginine ester; 6 = cinobufotoxin, resibufotoxin. Conditions: μBondapak C_{18} column; eluent, methanol–water (2:1), 1.5 ml/min; detection, 254 nm. (b) Fraction II: 1 = arenobufotoxin; 2 = resibufogenin 3-succinoylarginine ester; 3 = bufalin 3-adipoylarginine ester; 4 = bufalin 3-pimeloylarginine ester; 5 = bufalitoxin. Eluent, methanol–water (2:1), 1.0 ml/min. Other conditions as in (a). (c) Fraction III: 1 = gamabufotalin 3-succinoylarginine ester; 2 = gamabufotalin 3-adipoylarginine ester; 3 = gamabufotalin 3-pimeloylarginine ester; 4 = gamabufotalitoxin; 5 = bufalin 3-succinoylarginine ester. Conditions as in Fig. 2.

corresponding genin and the number of methylene in the dicarboxylic acid residue with the bufogenin and pregnadienolone derivatives. Satisfactory separations of some bufogenin 3-sulphates were also attained when a phosphate buffer solution was used as eluent (Fig. 8).

Several papers have dealt with the separation of conjugated steroids[14]. Previously used methods, however, have inevitable disadvantages, *i.e.*, lack of complete separation, necessity of derivatization prior to chromatography and limitation of the amount of sample. It should be noted that the present method is suitable for both the preparation and the analysis of bufotoxins and their related compounds in toad venom. Reversed-phase HPLC may be also applicable to the separation and determination of steroid hormone conjugates and cardiac glycosides.

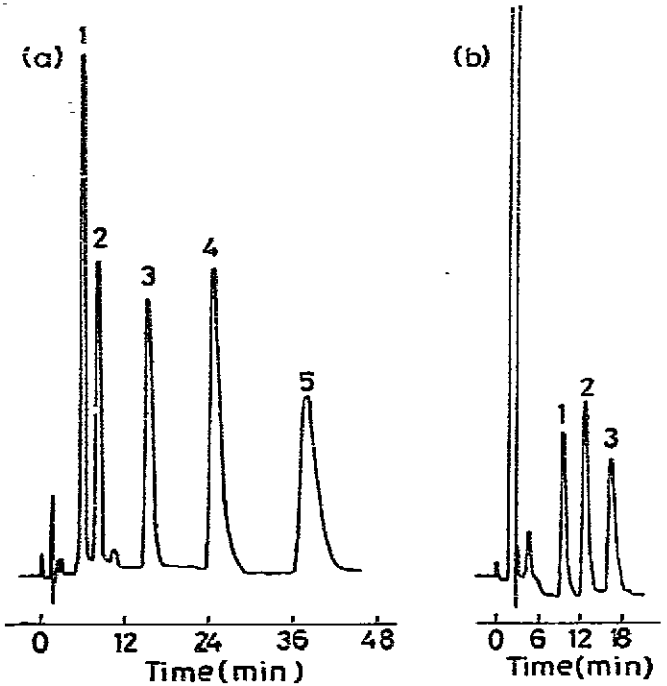

Fig. 5. (a) Separation of bufogenin dicarboxylic acid half-esters. 1 = Desacetylcinobufagin 3-hemisuccinate, sarmentogenin 3-hemisuberate, gamabufotalin 3-hemisuberate; 2 = arenobufagin 3-hemisuberate; 3 = bufotalin 3-hemisuberate; 4 = bufalin 3-hemisuberate; 5 = cinobufagin 3-hemisuberate. Conditions: μBondapak C_{18} column; eluent, methanol–0.02 *M* $NH_4H_2PO_4$ (2:1), 2 ml/min; detection, 254 nm. (b) Separation of desacetylcinobufagin 3-hemisuccinate (1), sarmentogenin 3-hemisuberate (2) and gamabufotalin 3-hemisuberate (3). Conditions: μBondapak C_{18} column; eluent, tetrahydrofuran–0.02 *M* $NH_4H_2PO_4$ (2:3), 1.5 ml/min; detection, 254 nm.

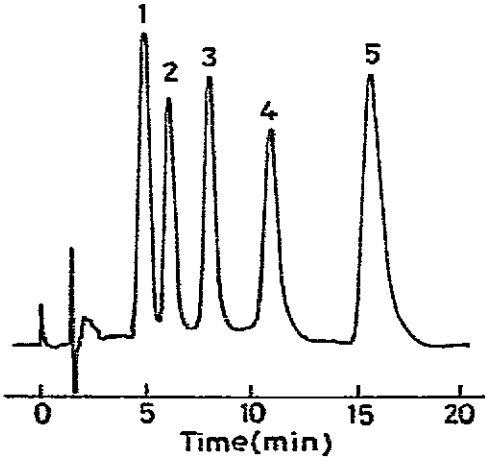

Fig. 6. Separation of bufalin dicarboxylic acid half-esters. 1 = 3-Hemisuccinate; 2 = 3-hemiglutarate; 3 = 3-hemiadipate; 4 = 3-hemipimelate; 5 = 3-hemisuberate. Conditions: μBondapak C_{18} column; eluent, methanol–0.02 *M* $NH_4H_2PO_4$ (2:1), 2 ml/min; detection, 280 nm.

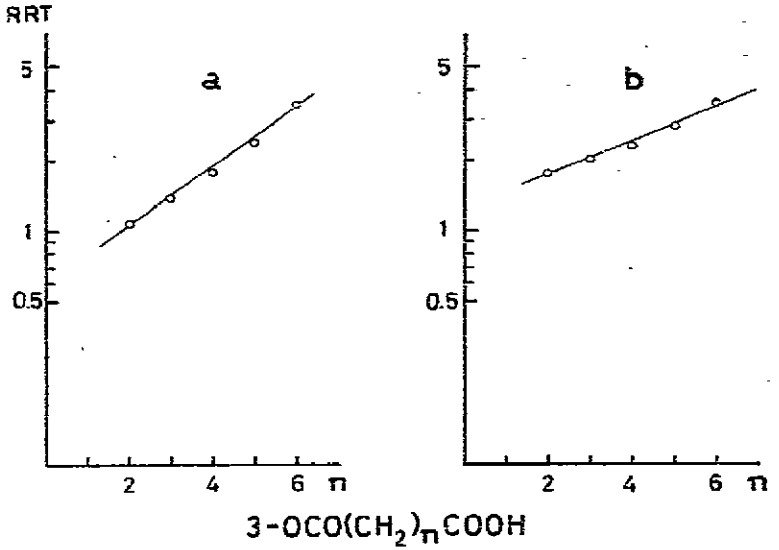

Fig. 7. Relationship between the relative retention time and the number of methylene groups in the dicarboxylic acid moiety. Conditions: μBondapak C_{18} column; eluent, (a) methanol–0.02 *M* $NH_4H_2PO_4$ (2:1), 2 ml/min, (b) tetrahydrofuran–0.02 *M* $NH_4H_2PO_4$ (4:5), 1.5 ml/min; detection, (a) 280 nm, (b) 254 nm. Retention time of internal standard: (a) bufalin, 2.3 min; (b) pregnadienolone, 6.1 min.

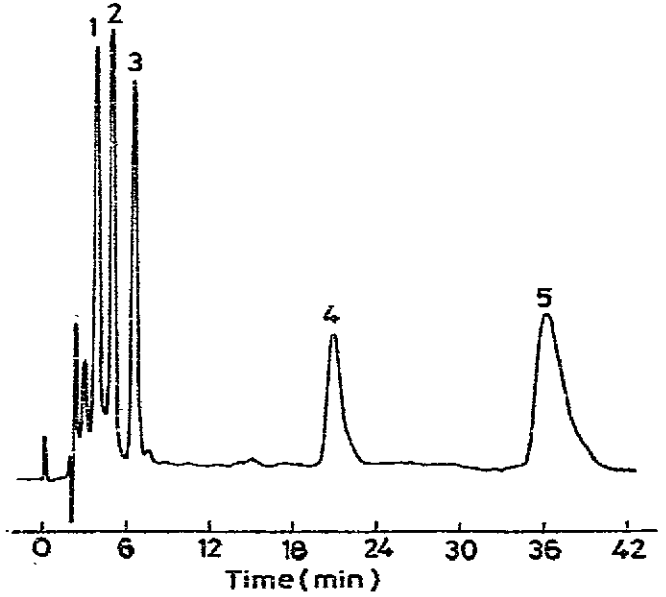

Fig. 8. Separation of bufogenin 3-sulphates. 1 = Sarmentogenin 3-sulphate; 2 = gamabufotalin 3-sulphate; 3 = arenobufagin 3-sulphate; 4 = bufotalin 3-sulphate; 5 = bufalin 3-sulphate. Conditions: μBondapak C_{18} column; eluent methanol–0.03 *M* $NH_4H_2PO_4$ (2:3), 1.5 ml/min; detection, 254 nm.

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