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Chromatography of crotamiton and its application to the determination of active ingredients in ointments[1]

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Abstract

Crotamiton, which is a mixture of *cis* and *trans* isomers, was investigated by several separation techniques. One of the HPLC modes, in which crotamiton eluted as a single peak, was selected for the determination of five active ingredients (crotamiton, prednisolone, glycyrrhetinic acid, dibucaine and chlorhexidine hydrochloride) in an ointment. The simultaneous determination was performed using isocratic reversed-phase mode within 20 min by employing an octyl (C_8) column and a mobile phase containing sodium dodecyl sulfate (SDS) and 2-propanol. The method was successfully applied to quality control and stability testing of the ointment. © 1997 Elsevier Science B.V.

Keywords: Crotamiton; HPLC; Isomers; Ointment; Prednisolone; Simultaneous determination

1. Introduction

Ointments used for skin infections contain small amounts of several active ingredients. Warbonplus Ointment® (Tanabe Seiyaku, in 1996), contains five active ingredients, namely crotamiton, prednisolone, glycyrrhetinic acid, dibucaine and chlorhexidine hydrochloride (see, Fig. 1 and Table 1) [1], and has been developed for use against various skin infections. For the determination of several active ingredients in formulations, HPLC has been widely used. In most cases, for formulations containing several active ingredients whose physico-chemical properties (especially, hydrophobicity) are quite different, several HPLC methods or gradient mode operation will be required for the assay of all active ingredients.

Among five active ingredients, crotamiton (crotonyl-*N*-ethyl-*o*-toluidine, see, Fig. 1), which is used as a scabicide, insecticide and antipruritic agent, has *cis*- and *trans*-isomers in approximate proportions of 5:95 [3], has been determined by supercritical fluid chromatography (SFC) [4], normal-phase HPLC [4] or reversed-phase HPLC [5]. The GC method is officially described in the Japanese Pharmaceutical Codex 1993 with a limit of *cis*-isomer (not more than 15%).

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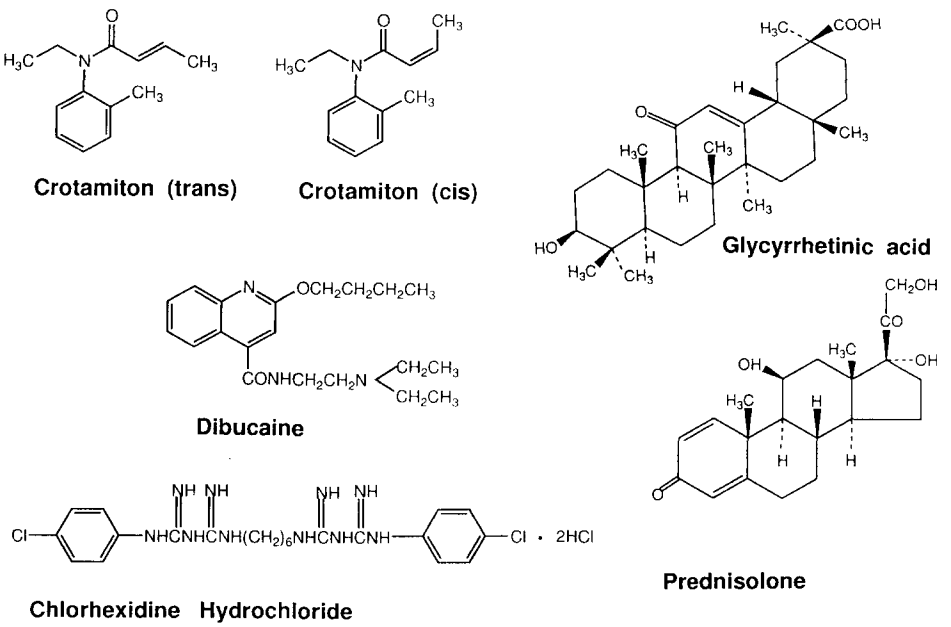

Fig. 1. Structures of active ingredients contained in the ointment.

In the quality control of ointments containing crotamiton, it will be better to use chromatographic conditions where crotamiton elutes as a single peak, although it is important to separate the isomers for the evaluation of the quality of crotamiton. One reason is simplicity and the other is correlated with the standard of crotamiton. That is, to evaluate crotamiton accurately, authentic *cis*- and *trans*-isomers must be used as standard. However, these are not available from commercial sources. In such case, each isomer may have to be prepared by preparative HPLC or a mixture of *cis*- and *trans*-isomer may be used as it is. In the latter case, relative sensitivity of each isomer must be checked. The isocratic mode is also favourable for the quality control method.

In this paper, chromatography of crotamiton is investigated by employing several separation techniques such as HPLC, GC and capillary electrophoresis (CE). Then, simultaneous HPLC determination of five active ingredients, which are neutral and basic, in the ointment is investigated. The assay method, where isocratic mode is used and crotamiton elutes as a single peak, is developed by manipulating the species of an organic solvent and the concentration of sodium dodecyl sulfate (SDS). The method is applied to the quality control and the stability study of the ointment. The significant advantages of the method are the simultaneous determination of five active ingredients by the isocratic mode and the coelution of crotamiton isomers. The isocratic mode is favourable as a routine quality control method, compared with the usual cases as mentioned above (i.e., gradient mode operation or several HPLC methods). The single peak of crotamiton isomers enables simple and easy calculation of its peak area (no necessity of summation of peak areas of *cis*-isomer and *trans*-isomer).

2. Experimental

2.1. Reagents and materials

Prednisolone of Japanese Pharmacopoeia grade was purchased from National Hygiene Research Laboratory (Tokyo, Japan). Chlorhexidine hydrochloride of Japanese Pharmacopoeia grade was obtained from Sumitomo Seiyaku, (Osaka,

Japan). Crotamiton of Japanese Pharmaceutical Codex grade was obtained from Kongo Kagaku, (Toyama, Japan). Glycyrrhetinic acid of Japanese Pharmaceutical Codex grade was purchased from Maruzen Seiyaku, (Osaka, Japan). Dibucaine was purchased from Nagase Fine Chemical, (Osaka, Japan). HPLC grade 2-propanol (2-PA), acetonitrile (ACN) and tetrahydrofuran (THF) were obtained from Katayama Kagaku Kogyo, (Osaka, Japan). Methanol (MeOH), hexane, phosphoric acid, sodium dihydogenphosphate and formic acid of reagent grade were obtained from Katayama Kagaku. Sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) for ion-pair chromatography was purchased from Nacalai Tesque(Kyoto, Japan). All other reagents used were of analytical reagent grade from Katayama Kagaku Kogyo. Purified water was prepared with a Millipore RO-60 water system (Millipor Japan, Tokyo, Japan). Phenanthrene of analytical reagent grade from Tokyo Kasei Kogyo, (Tokyo, Japan) was used as an internal standard (IS) substance.

2.2. HPLC instruments

A Shimadzu LC-6A or LC-10A system (Kyoto, Japan) equipped with a Shimadzu SPD-6A or SPD-10A UV spectrophotometric detector were used for the HPLC determination of active ingredients in the ointment and the isomer ratio of crotamiton. The sample solution was injected by using a Shimadzu SIL-6B or SIL-10A automatic injector. Peak integration was carried out with a Shimadzu Chromatopac C-R5A or C-R7A data-processor. HPLC columns used were Develosil ODS-HG-5 (5 μm, 6 mm i.d. × 100 mm or 150 mm, Nomura Chemicals, Aichi, Japan), Develosil $5C_8$ (5 μm, 4.6 mm i.d. × 150 mm, Nomura Chemicals), Zorbax RX-C_8 (5 μm, 4.6 mm i.d. × 150 mm, Dupont, Wilmington, USA) and ULTRON ES β-CD (5 μm, 6 mm i.d. × 150 mm, Shinwa-kako, Kyoto, Japan).

2.3. GC instrument

The GC was carried out according to the authorized procedure [3], using a Shimadzu GC-14A system. The column was a glass-column of 3 mm in inner diameter and 2 m in length, packed with Chromosorb W (177–250 μm) coated 10% polyethylene glycol 20 M (GL Science, Tokyo, Japan). The column temperature was maintained at 210°C by the oven. The flame ionization detector (FID) was operated at 250°C. The sample solution (2 μl) was injected through the septum injector at 240°C. The carrier gas nitrogen was flowed at ca. 20 ml min^{-1}.

2.4. CE instrument

CE experiments were performed on a Beckman P/ACE system 5510 equipped with a photodiode array detector (Beckman Instruments, Fullerton, CA, USA). An uncoated capillary tube (75 μm i.d., effective length 35 cm) was used as a separation tube. The capillary tube was thermostated at 20°C by a liquid coolant. The detection wavelength was set at 254 nm. The applied voltage was 20 kV and the sample was introduced by the pressure mode (0.5 psi, 3–9 s). Instrument control was performed with a personal computer (COMPAQ ProLinea 4/33).

2.5. Structural analysis

A mass analysis of crotamiton isomers was performed by using an M-80A mass spectrometer (Hitachi, Ibaragi, Japan) in electron impact mode at 70 eV. A NMR measurement of the isomers was performed by using Bruker AC-200 type (200 MHz, CA, USA). A Shimadzu SPD-M6A photodiode array UV-VIS detector was used to monitor the UV spectra of the isomers.

Table 1
Active ingredients in the ointment (%)

Active ingredient	Abbreviation	Content
Prednisolone	PRE	0.25
Crotamiton	CRO	5
Dibucaine	DIB	0.25
Chlorhexidine hydrochloride	CHL	0.25
Glycyrrhetinic acid	GLY	0.5

2.6. Extraction procedure of active ingredients

About 1 g of the ointment was weighed accurately into the separating funnel and 10 ml of hexane and 20 ml of the extraction solvent, which is a mixture of MeOH/water/formic acid = 16/3/1, was added to the funnel. The funnel was shaken vigorously for 10 min and allowed to stand for 30 min. The lower layer was transferred to a 50 ml volumetric flask. This extraction process was carried out twice with 10 ml of the extraction solvent and each lower layer was added to the same flask. Exactly 5 ml of an IS solution, which was prepared by dissolving 25 mg of phenanthrene in 100 ml of methanol, was added to the flask and the extraction solvent was added to the flask to make the volume. The solution was diluted with the mobile phase to one-tenth, and this solution was used as the sample solution. A total of 20 μl of the solution was injected to HPLC.

2.7. Preparation of standard solutions

Each authentic sample, whose amounts are nearly equivalent to 10 times the labelled amounts, was weighed accurately into a 100 ml volumetric flask and the extraction solvent was added to the flask to make the volume. Exactly 10 ml of the solution and exactly 5 ml of the IS solution were added to a 50-ml volumetric flask and the extraction solvent was added to make the volume. The solution was diluted with the mobile phase to one-tenth, and this solution was used as the standard solution. Of the solution, 20 μl was injected to HPLC.

2.8. HPLC conditions for the assay of active ingredients in the ointment

The assay of active ingredients contained in the ointment was carried out isocratically at 40°C on Zorbax RX-C_8 (5 μm, 4.6 mm $\phi \times 15$ cm) using a mobile phase consisting of 2-PA/0.05 M phosphate buffer (pH 3.0) containing 0.3% SDS = 40/60 (V/V). The pH of the buffer was adjusted by adding phosphoric acid to a 0.05 M sodium dihydrogenphosphate solution containing 0.3% SDS. The flow rate was 1.0 ml min^{-1}. The detection wavelength was 270 nm.

Table 2
Contents of *cis*-isomer of crotamiton determined by GC and HPLC

Crotamiton lot No.	GC method (%)	HPLC method (%)[a]
060	2.31	2.10
161	2.16	2.04
100	2.07	1.99
110	2.49	2.11

[a]HPLC conditions: detection wavelength, 270 nm; column, Develosil ODS-HG-5 (4.6 mm $\phi \times 150$ mm); Flow rate, 1.0 ml min^{-1}.

3. Results and discussion

3.1. Chromatography of crotamiton

Crotamiton is a mixture of *trans*- and *cis*-isomers [2]. The ratio of the isomers is regulated with a limit of *cis*-isomer (not more than 15%) [3]. The *cis*/*trans* ratio was determined by the GC method according to the official method [3]. The results of four batches are summarized in Table 2. It was found that commercial crotamiton obtained all contained *cis*-isomer (ca. 2–3%). This means that pure *trans* (or *cis*) crotamiton can not be obtained from commercial sources. A GC chromatogram of crotamiton is shown in Fig. 2A, where *cis*-isomer eluted faster than the *trans*-isomer. The elution order observed in GC was the same as in SFC and HPLC of normal-phase mode [4]. Separation of the isomers has also been achieved by reversed-phase HPLC [5]. In Ref. [5], ACN and ODS column were employed for the separation and *trans*-isomer eluted faster than *cis*- isomer in the reversed-phase mode.

Separation of crotamiton was also investigated by CE employing micellar electrokinetic chromatography (MEKC) [6,7] because crotamiton is an electrically neutral compound. Crotamiton migrated near the peak of the marker of the micelle when a micellar solution (SDS or CTAB) was used alone as a running buffer solution. This means that crotamiton is hydrophobic. Relatively fast migration of crotamiton was obtained by adding an organic solvent to the micellar solution. In MEKC with SDS or CTAB, a single peak was obtained from crotamiton as shown in Fig. 2B.

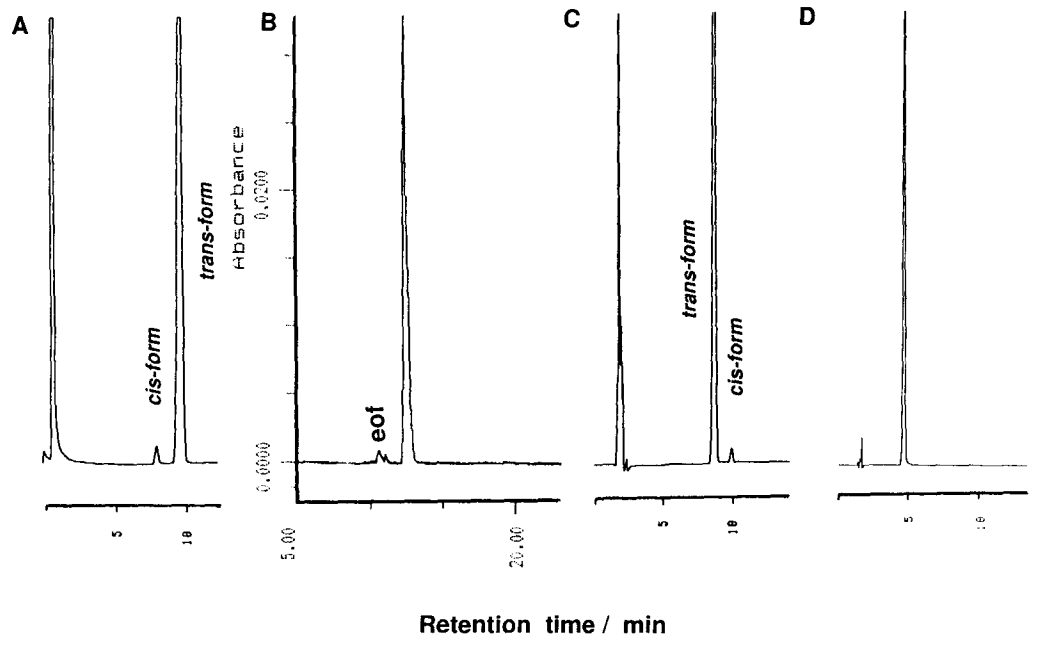

Fig. 2. Separation of isomers of crotamiton by (A) GC, (B) CE, (C) HPLC (ODS—THF/ACN), (D) HPLC(C_8—2-PA). (A)GC condition: column, Chromosorb W coated 10% polyethylene glycol 20 M (3 mm $\phi \times 2$ m); column temperature, 210°C; carrier gas, nitrogen gas; flow rate, 20 ml min^{-1}; detector temperature, 250°C; injector temperature, 240°C. (B) CE condition: uncoated capillary (50 μm $\phi \times 35$ cm); column temperature, 20°C; running buffer solution, 20 mM phosphate-borate buffer (pH 9.0) containing 25 mM SDS and 50% methanol; applied voltage, 20 kV; detection, 254 nm. eof: electroosmotic flow. (C) HPLC condition: column, Develosil ODS-HG-5 (5 μm, 4.6 mm $\phi \times 15$ cm); column temperature, 40°C; mobile phase, 0.1% phosphoric acid/ACN/THF = 26/7/7; flow rate, 1.0 ml min^{-1}; detection, 270 nm. (D) HPLC condition: column, Zorbax RX-C_8 (5 μm, 4.6 mm $\phi \times 15$ cm); column temperature, 40°C; mobile phase, 0.05 M phosphate buffer (pH 3.0)/2-PA = 60/40; Flow rate, 1.0 ml min^{-1}; detection, 270 nm.

HPLC separation of crotamiton in reversed-phase mode was investigated in detail by employing an ODS and C_8 column. Separation of isomers of crotamiton can be manipulated mainly by changing the mobile phase in reversed-phase HPLC. The effect of an organic solvent and a column on the separation of the isomers are summarized in Table 3. The concentration of an organic solvent and the flow rate were adjusted so that the *trans*-isomer elutes at around 5 min. THF gave the best separation of isomers among four organic solvents investigated. ACN also gave the sufficient separation of the isomers in use of an ODS column. An ODS column was superior to a C_8 column for the separation of the isomers, judged from the resolution (R_s) values.

Other than ODS and C_8, separation of the isomers was investigated by using a special column. Large separation selectivity of the isomers was obtained by employing β-cyclodextrin (β-CD) immobilized column, ULTRON ES-β-CD. This immobilized column is usually used in reversed-phase mode for the enantiomer separation [8,9]. The resolution R_s of 8.3 was obtained with the mobile phase of 0.1% phosphoric acid/MeOH = 7/3. *Trans*-isomer eluted faster (5.64 min) than the *cis*-isomer (13.5 min) as in an ODS and C_8 columns. The elution order in the reversed-phase mode including β-CD column means that *cis*-isomer is more hydrophobic. It was found that crotamiton can be eluted as one peak or two peaks by choosing the appropriate HPLC conditions.

Among the conditions in Table 3, Develosil ODS-HG-5(4.6 mm $\phi \times 150$ mm) with a mobile phase of 0.1% phosphoric acid/ACN/THF = 26/7/

Table 3
Effect of an organic solvent on the separation of isomers of crotamiton

Column	Organic solvent (%)	Flow rate (ml min^{-1})	t^{a}_{Rt} (min)[a]	t_{Rc} (min)[b]	R_s
Develosil ODS-HG-5	THF 30	0.75	5.38	6.62	3.82
(4.6 mm $\phi \times 100$ mm)	ACN 45	1.0	5.28	5.83	1.97
	MeOH 60	0.9	5.87	6.24	—
	2-PA 35	0.8	5.78	6.32	1.40
	THF/ACN (1:1) 35	1.0	5.3	6.05	2.43
Zorbax RX-C_8	THF 30	1.4	6.68	7.88	3.51
(4.6 mm $\phi \times 150$ mm)	ACN 45	1.0	6.37	6.37	0
	MeOH 65	1.0	5.45	5.45	0
	2-PA 40	1.0	4.52	4.52	0
	THF/ACN (1:1) 35	1.0	7.34	7.97	1.85
Develosil $5C_8$	THF 30	1.4	7.37	8.83	3.48
(4.6 mm $\phi \times 150$ mm)	ACN 45	1.0	7.63	8.12	1.51
	MeOH 60	1.0	8.40	8.40	0
	2-PA 40	1.0	4.43	4.43	0

[a]Retention time of *trans*-isomer of crotamiton.
[b]Retention time of *cis*-isomer of crotamiton.

7 was selected for the determination method of isomers in crotamiton. A mixture of ACN and THF (1:1) was selected as an organic modifier of the mobile phase because of the sufficient resolution (> 2.0) and no interruption of other peaks. The slow eluted peaks appeared in use of THF alone as an organic modifier in the continuous runs of actual sample solutions. A chromatogram by the condition is shown in Fig. 2C. The contents of *cis*-isomer (minor peak) in four batches determined by the condition in Fig. 2C are summarized in Table 2. The results by HPLC (UV detection) were identical with those by GC (FID detection). The method in Fig. 2C was also successful for the isomer determination of crotamiton in other commercial ointments. Chromatograms in the *cis*/*trans* determination of crotamiton in ointments are shown in Fig. 3. About 2% *cis* crotamiton was detected in other commercial ointments. Some parabens were also detected in Fig. 3. By the conditions in Fig. 2C, relatively fast separation of *cis*- and *trans*-isomers was achieved without coelution of the other compounds and slow eluted compounds. In the latter case, continuous operation of HPLC determination is unsuccessful and requires long analysis times.

Presuming that FID detection in GC gives the same sensitivity to the isomers, the results in Table 2 indicate that *cis*-isomer and *trans*-isomer have the same sensitivity (molar absorption coefficient) in HPLC. That is, there is no problem concerning the evaluation of crotamiton as a single peak. Thus, 2-PA and a C_8 column were selected for the assay of the ointment containing crotamiton. An HPLC chromatogram of crotamiton using a mobile phase of 0.05 M phosphate buffer (pH 3.0)/2-PA = 60/40 is shown in Fig. 2D. Finally, both peaks in Fig. 2C were collected and analyzed by UV, MS and NMR to identify the isomeric structure (*cis* or *trans*). The data indicated that the major peak (faster eluted peak) was *trans*-isomer.

3.2. Separation of five active ingredients

The HPLC conditions for the separation of five active ingredients were investigated with Zorbax RX-C_8 and 2-PA. The C_8 column and 2-PA were selected to obtain a single crotamiton peak and a relatively shorter analysis time than on an ODS column. For neutral or hydrophobic compounds, retention in reversed-phase mode is usually manipulated by the concentration of an organic

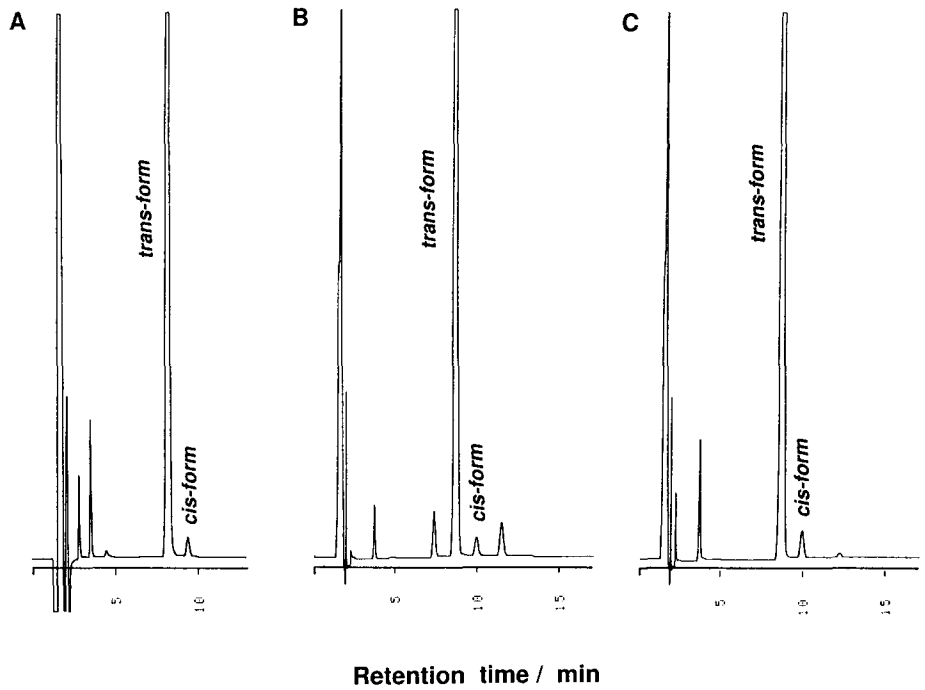

Fig. 3. Determination of *cis/trans* ratio of crotamiton in ointments. (A) Warbonplus (B) Commercial ointment from M company. (C) Commercial ointment from U company. Conditions are the same as in Fig. 2C.

modifier. In this ointment, there are three neutral active ingredients at pH 3, namely crotamiton, predonisolone and glycyrrhetic acid. Separation of the three neutral drugs was investigated by changing 2-PA concentration and 40% of 2-PA was selected. At this concentration, predonisolone, crotamiton and glycyrrhetic acid eluted at approximately 3, 5 and 18 min, respectively.

Next, retention of the other two basic drugs was investigated. Other than organic solvents, retention of compounds, especially for ionic compounds, in reversed-phase HPLC can be manipulated through additives such as ion-pairing agent. The effects of the addition of SDS on the separation of five active ingredients are shown in Fig. 4. Without SDS, these two basic drugs eluted close to prednisolone. By increasing SDS concentration, retention of both chlorhexidine and dibucaine increased, although those of the neutral drugs did not change. The selected mobile phase composition (0.3% SDS) was described in the experimental section. Phenanthrene, which eluted between the peak of dibucaine and that of chlorhexidine, was selected as an IS substance. A chromatogram of the standard solution containing the IS substance is shown in Fig. 5. One analysis was performed isocratically within 20 min and each component migrated with almost the same intervals under the conditions. Under the other HPLC condition, which is suited for one of five active ingredients (typically, simple reversed-phase mode), some eluted fast (almost at the solvent front peak) and some did not elute. That is, simultaneous separation was not achieved.

3.3. Extraction of active ingredients from the ointment

The extraction of active ingredients from the ointment matrix was carried out according to the procedure described in the experimental section. The efficiency of extraction at each repetition process is summarized in Table 4. All active ingre-

dients except for crotamiton were extracted perfectly by one extraction. It was necessary for the quantitative recovery of crotamiton to repeat the extraction process three times. The acidic conditions were required for the perfect extraction of dibucaine and chlorhexidine. The extractability of the two basic active ingredients in one extraction process using a solution MeOH/water = 16/3 was between 90–95%, although prednisolone and glycyrrhetinic acid were almost extracted 100% by the solvent.

3.4. Method validation

The linearity of five active ingredients was investigated in 60–140% of the assay concentration. All calibration graphs obtained by the IS method passed through the origin with the correlation values of more than 0.999 (crotamiton $r = 0.9999$, chlorhexidine $r = 0.9999$, prednisolone $r = 0.9999$, glycyrrhetinic acid $r = 0.9996$, dibucaine $r = 0.9994$). Recovery testing of each active ingredient was investigated by adding each authentic sample (90–110% of the labelled amount) to the placebo mixture, which was prepared in the absence of the

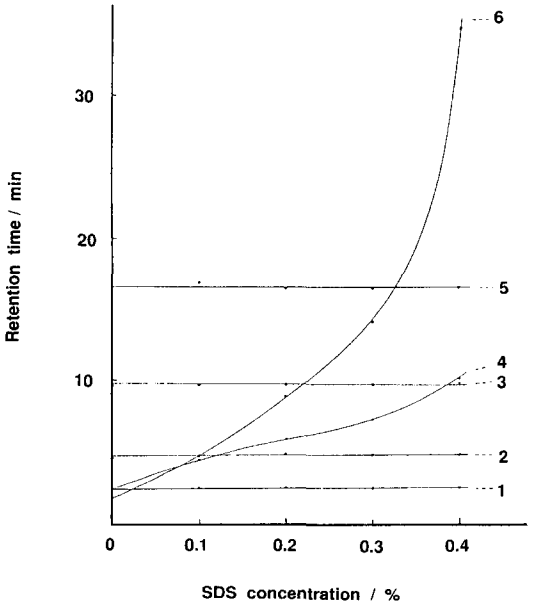

Fig. 4. Effect of SDS concentration on the retention of five active ingredients. Solutes number, 1 = prednisolone, 2 = crotamiton, 3 = phenanthrene (IS), 4 = dibucaine 5 = glycyrrhetinic acid, 6 = chlorhexidine. Conditions: column, Zorbax RX-C_8 (5 μm, 4.6 mm $\phi \times 15$ cm); column temeprature, 40°C; moble phase, 0.05 M phosphate buffer (pH 3.0)/2-PA = 60/40 with SDS; Flow rate, 1.0 ml min^{-1}; detection, 270 nm.

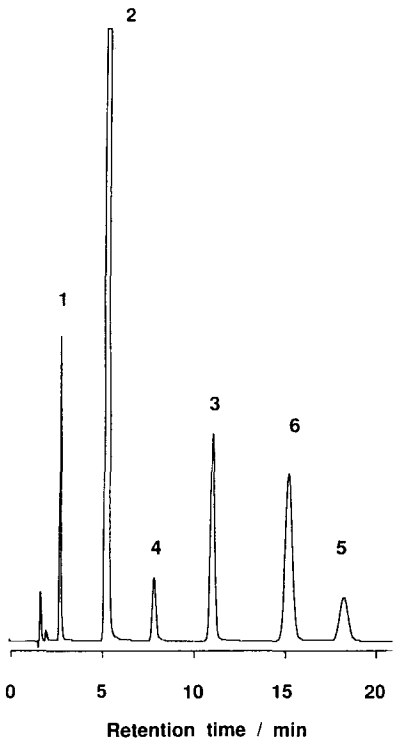

Fig. 5. Separation of five active ingredients contained in Warbonplus ointment. Conditions: column, Zorbax RX-C_8 (5 μm, 4.6 mm $\phi \times 15$ cm); column temperature, 40°C; mobile phase, 2-PA/0.05 M phosphate buffer (pH 3.0) containing 0.3% SDS = 40/60; flow rate, 0.8 ml min^{-1}; detection, 270 nm. Solutes numbers are indicated with the same number as in Fig. 4.

Table 4
Extraction efficiency of five active ingredients in the ointment (%)[a]

Repetition	PRE	CRO	DIB	CHL	GLY
1	99.9	92.8	100.3	99.7	102.2
2	0.9	7.9	0	0	0
3	0	1.1	0	0	0
Total	100.8	101.6	100.3	99.7	102.2

[a]Abbreviations of the active ingredients are shown in Table 1.

Table 5
Recovery testing of five active ingredients (%)[a]

Repetition	Added value (%)	PRE	CRO	DIB	CHL	GLY
1	90	101.5	98.4	99.4	99.3	99.2
2	90	100.4	99.0	100.4	99.8	100.2
3	100	101.4	100.8	104.2	102.7	101.6
4	100	101.7	101.7	102.0	101.6	101.4
5	100	99.6	98.3	101.6	99.2	98.6
6	110	100.5	99.7	101.8	100.0	100.3
7	110	101.5	100.2	100.0	99.4	99.3
Average recovery		100.9	99.7	101.3	100.3	100.1
R.S.D. (%)		0.74	1.27	1.58	1.34	1.16

[a]Abbreviations of the active ingredients are shown in Table 1.

active ingredient. These samples were treated according to the extraction process described in the experimental section and the content of each active ingredient was determined by the method. The results are summarized in Table 5. Almost 100% recovery was obtained, showing the sufficient extraction. No interference peak was observed from the sample solution prepared by the placebo mixture.

Stability of the sample solutions and the reproducibility of the method were also investigated. No degradation product appeared in the sample solution stored at room temperature (approximately 25°C) for 1 day. The reproducibility (R.S.D.) of the assay method, including the HPLC reproducibility, was around 1% (see, Table 6). These values were almost the same as in those obtained for the other ointments. However, these were larger compared with those in other HPLC determinations of active ingredients in tablets etc. This may ascribed to the repeated extraction process ($n = 3$) in this ointment.

3.5. Application to the quality control and the stability study of the ointment

The method was applied to the quality control of the ointment. Assay results of one of three batches, which were used for the stability study, are summarized in Table 6. The stability study of the ointment was performed at 25°C and 40°C/75% R.H. The results of the ointments stored at 40°C/75% R.H. for 6 months are summarized in Table 7. The assay results of the ointments stored at 25°C for 4 years was between 93 and 100% of the labelled amounts. These results indicate that the ointment has a good stability.

Table 6
Contents of active ingredients in the ointment (%) [a]

Repetition	PRE	CRO	DIB	CHL	GLY
1	98.0	100.9	100.4	101.2	100.3
2	96.3	99.7	99.8	100.7	99.6
3	97.3	100.1	100.6	101.4	100.7
4	95.4	100.3	98.5	99.5	99.7
5	96.6	102.9	97.0	100.2	101.1
Average	96.7	100.8	99.3	100.6	100.3
R.S.D. (%)	1.02	1.25	1.51	0.77	0.60

[a]Abbreviations of the active ingredients are shown in Table 1.

Table 7
Stability study of the ointment stored at 40°C and 75% R.H. for 6 months (%)

Repetition	PRE	CRO	DIB	CHL	GLY
1	94.9	100.3	99.8	101.7	98.0
2	93.3	99.3	96.7	99.0	97.1
3	94.3	101.0	101.5	100.4	97.4
Average	94.2	100.2	99.3	100.4	97.5
Residue	97.4	99.4	100.0	99.8	97.2

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