ELSEVIER

Journal of Chromatography A, 678 (1994) 69–76

JOURNAL OF CHROMATOGRAPHY A

Determination of (2*S*, 3*S*, 5*R*)-3-methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (YTR-830H) and piperacillin in pharmaceutical preparations by high-performance liquid chromatography

Tsuyoshi Tsukamoto*, Takanori Ushio

Department of Analytical Research, Pharmaceutical Research Laboratory, Taiho Pharmaceutical Co., Ltd., 224-2, Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-01, Japan

(First received October 20th, 1993; revised manuscript received March 29th, 1994)

Abstract

A high-performance liquid chromatographic method using a wavelength-scanning system was developed for the determination of (2*S*, 3*S*, 5*R*)-3-methyl-7-oxo-3-(1*H*-1, 2, 3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (**I**) and piperacillin (PIPC) in pharmaceutical preparations. **I** and PIPC were determined using a reversed-phase column with a mixture of 10 m*M* tetra-*n*-butylammonium hydroxide and 5 m*M* K_2SO_4 (pH 4.1), acetonitrile and methanol (1000:300:25) as the mobile phase. Addition of SO_4^{2-} to the mobile phase was useful for the separation of related substances and improving the peak tailing of **I** and PIPC. This mobile phase was also suitable for the determination of PIPC, **I** and their degradation products in pharmaceutical preparations. Detection was based on the ultraviolet absorption of **I** at 220 nm and of PIPC at 270 nm using a wavelength-scanning system. The calibration graphs were linear over the ranges 0–7.5 μg for **I** and 0–30 μg for PIPC. The precisions (relative standard deviations of six analyses) of **I** and PIPC were 0.45% and 0.33%, respectively.

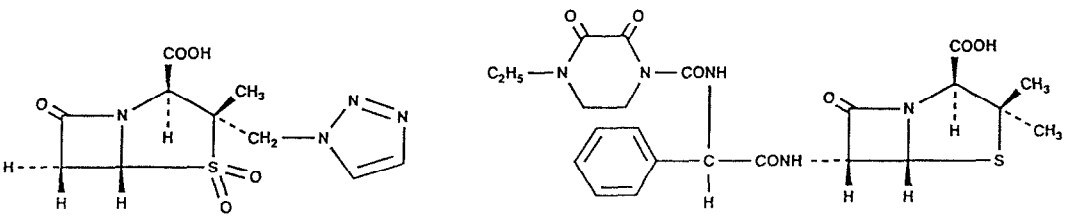
1. Introduction

The combined use of β-lactam antibiotics with β-lactamase inhibitor was effective against β-lactamase-producing antibiotic-resistant strains. Clavulanic acid and sulbactam have been developed as potent β-lactamase inhibitors [1,2] and have been commercialized world-wide. A novel β-lactamase inhibitor, YTR-830H, (2*S*, 3*S*, 5*R*)-3-methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (**I**) (Fig. 1), was introduced by Micetich *et al.* [3]. Various investigations of **I** with β-lactam antibiotics [4–12] showed that the combined use of **I** with piperacillin (PIPC) was most effective against various β-lactamase-producing bacteria [13].

An analytical method was required for the combined formulation of **I** and PIPC. At first **I** and PIPC in pharmaceutical preparations were

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00320-9



I PIPC

Fig. 1. Structures of I and piperacilln (PIPC).

separately determined by using individual HPLC methods. However, a simple and reliable HPLC method was required for pharmaceutical preparations, involving routine quality control and stability assays. I and PIPC have no UV absorption maxima above 210 nm. Degradation of I [14,15] and PIPC [16] yielded many structurally related compounds. This paper describes an HPLC method for the simultaneous determination of I and PIPC using ion-pairing and wavelength-scanning techniques. The method was successfully applied to the separation of degradation products of I and PIPC in a stability study of pharmaceutical preparations.

2. Experimental

2.1. Chemicals and reagents

Methanol, acetonitrile and tetra-*n*-butylammonium hydroxide (TBAH) solution (0.5 *M*) of HPLC-grade were obtained from Wako (Osaka, Japan). Tetra-*n*-butylammonium hydrogensulphate (TBAHS) of analytical-reagent grade was purchased from Aldrich (Steinheim, Germany). Diammonium hydrogenphosphate, potassium sulphate and phosphoric acid of analytical-reagent grade were purchased from Wako. Water purified with a Milli-Q water-purification system (Millipore, Bedford MA, USA) was used in all procedures. Combined formulations of I and PIPC, with a mass ratio of 1:4, were prepared by Taiho Pharmaceutical (Tokyo, Japan).

2.2. Instrumentation

The HPLC system consisted of a Model 600E multi-solvent delivery system, a Model 468 programmable multi-wavelength detector and a WISP Model 712 autosampler (Waters Chromatography Division, Millipore, Milford, MA, USA) or a Model LC-7A multi-solvent delivery system, a Model SPD-6AV programmable multi-wavelength detector (Shimadzu, Kyoto, Japan) and a WISP model 712 autosampler (Waters Chromatography Division). A Model C-R4A integrator (Shimadzu) was used to record chromatograms and calculate peak areas. A Waters Model 990J photodiode-array detector was used when assessing the homogeneity of the YTR-830H and PIPC peaks.

2.3. Columns

The analytical columns used were as follows: column A, Ultron phenyl; column B, Ultron N-C_{18}; column H, Ultron ODS-X; and column I, Ultron N-C_8 (all from Shinwa Chemical Industries, Kyoto, Japan); column C, TSK-Gel ODS 80TM (Tosoh, Tokyo, Japan); column D, Wakopak 5C_{18} (Wako); column E, Unisil Q C_{18} (GL Science, Tokyo, Japan); column F, Capcellpak C_{18} (Shiseido, Tokyo, Japan); and column G, Cosmosil 5C_{18} (Nacalai Tesque, Osaka, Japan). All packing materials were made from silica gels except for the Capcellpak C_{18}, which is made from a polymer-coated silica gel. All materials were packed into a 150 mm × 4.6 mm I.D. stainless-steel column.

2.4. Sample preparation

Known amounts of I and PIPC were dissolved in mobile phases or a mixture of acetonitrile and mobile phase. The concentrations of I and PIPC were adjusted to about 0.5 and 2.0 mg/ml, respectively.

2.5. HPLC analysis

A 10-μl aliquot of the sample solution was injected on to a column. Other chromatographic conditions are given in the figures and tables. The influence of the mobile phase pH and concentrations of ion-pairing reagent, SO_4^{2-} and methanol on capacity factors, peak tailing, separation factor and the resolution of I and PIPC were examined as described under Results and Discussion. Chromatographic parameters such as tailing and resolution were calculated according to the Pharmacopoeia of Japan [17]. The concentrations of I and PIPC in the sample were calculated from a calibration graph of concentration *vs.* peak area.

3. Results and discussion

3.1. Influence of mobile phase pH

The retention of I and PIPC was investigated with and without addition of TBAHS as an ion-pairing reagent. The ion-pairing reagent was useful for the simultaneous determination of I and PIPC under isocratic conditions. When the pharmaceutical preparation was stored in heated conditions, degradation of I and PIPC yielded many structurally related compounds. Therefore, we focused just on a main unknown degradation product. First, 5 m*M* TBAHS was used as an ion-pairing reagent. The pH of the mobile phase was adjusted with phosphoric acid. The relationships between the capacity factors (k') of I, PIPC and the main unknown degradation product and the pH of the mobile phase were investigated at pH 3.8, 5.3 and 7.0. When the pH of the mobile phase was increased to 7.0, the k' values of I and PIPC decreased. The separation of degradation products from I and PIPC was then investigated under acidic conditions. The chromatograms obtained with the use of TBAH and TBAHS were almost identical. However, for the separation of degradation products from PIPC, TBAH was better than TBAHS. In subsequent investigations 6.5 m*M* TBAH was used as the ion-pairing reagent.

The relationships between the capacity factors and the pH of the mobile phase at pH 3.1, 4.0 and 5.0 are shown in Table 1. As the pH of the mobile phase was increased to 5.0, the k' value of the unknown degradation product increased. The capacity factor of I remained unchanged, and that of PIPC was maximum at pH 4.0. Taking into account the separation of I and PIPC and the separation of degradation products from I and PIPC, the optimum pH of mobile phase was about 4 for the simultaneous determination of I and PIPC.

Table 1
Influence of the mobile phase pH on the capacity factors of I, PIPC and the main degradation product

Compound	Capacity factor (k')		
	pH 5.0	pH 4.0	pH 3.1
I	1.36	1.53	1.55
PIPC	4.50	4.89	4.18
Main degradation product	3.33	2.84	2.53
α[a]	1.35	1.72	1.65

I and PIPC was stored at room temperature for 8 days. Chromatographic conditions: column, Ultron N-C_8; mobile phase, mixture of water containing 6.5 m*M* TABH (pH is adjusted with phosphoric acid) and acetonitrile (100:45); column temperature, room temperature; flow-rate, 0.7 ml/min; detection wavelength, 220 nm. Mean results ($n = 3$).
[a] Separation factor of PIPC and main degradation product.

3.2. Column selection

Various commercially available reversed-phase columns were examined for the separation of I and PIPC by using a mobile phase (pH 4.1, adjusted with phosphoric acid) containing 6.5 m*M* TBAH as an ion-pairing reagent. Degradation of pharmaceutical preparations yielded many structurally related compounds. The separation of degradation products from I and PIPC was investigated using the degraded sample. The capacity factors and tailing factors of I and PIPC and the resolution between I and PIPC are shown in Table 2. All the reversed-phase columns were efficient for the separation of I and PIPC. However, the peaks were broad with the use of phenyl and polymer-coated ODS columns. Columns G and H were better for the separation

Table 2
Capacity factors (k'), tailing factors (T) and resolution (R_s) of I and PIPC on various columns

Column	I		PIPC		R_s
	k'	T	k'	T	
A	1.40	1.21	5.63	1.62	14.9
B	1.25	1.58	4.12	2.60	15.1
C	2.35	1.72	8.26	2.94	18.1
D	1.63	—	5.29	—	—
E	1.75	1.22	5.83	2.04	13.1
F	1.12	1.67	3.28	2.79	9.1
G	1.43	1.88	4.51	3.12	10.6
H	1.19	1.70	3.52	2.73	11.0
I	1.24	1.62	3.79	2.36	12.0

Chromatographic conditions: mobile phase, mixture of 6.5 m*M* TBAH (pH 4.1, adjusted with phosphoric acid) and acetonitrile (1000:450 or 1000:400); column temperature, room temperature; flow-rate, 0.7 ml/min. Column H = Ultron ODS-X. Mean results ($n = 3$).

of degradation products from I and PIPC. Taking into account the analytical time and tailing factor, column H (Ultron ODS-X, 5 μm particle size, 16% carbon content) was selected for the simultaneous determination of I and PIPC.

3.3. Influence of concentration of ion-pairing reagent

Table 3 shows the influence on the capacity factors and tailing factors of I and PIPC and the resolution between I and PIPC of the concentration of an ion-pairing reagent with a mobile phase of pH 4.1. At pH 4.1, changes in the TBAH concentration in the mobile phase had almost no effect on the retentions of I and PIPC. However, an increase in the concentration of the ion-pairing reagent gave an increase in resolution between I and PIPC and a decrease in the peak tailing of I and PIPC. In the degraded sample, the degradation products were not separated from I at a concentration of 3 m*M* TBAH and PIPC at a concentration of 15 m*M* TBAH. Therefore 10 m*M* TBAH was selected.

Table 3
Influence of concentration of TBAH on the capacity factors (k'), tailing factors (T) and resolution (R_s)

Concentration of TABH (m*M*)	I		PIPC		R_s
	k'	T	k'	T	
3	1.94	1.79	6.35	3.95	13.2
10	1.76	1.60	6.14	3.25	16.3
15	1.72	1.55	6.22	2.95	17.6

Chromatographic conditions: column, Ultron ODS-X; mobile phase, mixture of each concentration of TBAH (pH 4.1, adjusted with phosphoric acid) and acetonitrile (10:4); column temperature, room temperature; flow-rate, 0.7 ml/min; detection wavelength, 220 nm. Mean results ($n = 3$).

3.4. Influence of SO_4^{2-} ion and methanol

The influence of the SO_4^{2-} ion on the tailing of I and PIPC at concentrations of 2, 5 and 10 m*M* K_2SO_4 in the mobile phase was investigated. Addition of 2–10 m*M* SO_4^{2-} ion to the mobile phase gave significant improvements to the PIPC peak tailing and the resolution between I and PIPC. The tailing factor of PIPC was dependent on the concentration of SO_4^{2-}. When the concentration of SO_4^{2-} in the mobile phase was increased from 2 to 10 m*M*, the tailing of PIPC decreased from 2.88 to 2.23 and the retention of PIPC decreased. This gave a change in resolution between I and PIPC. In the degraded sample, the degradation products were not separated from I at a concentration of 10 m*M* SO_4^{2-} and from PIPC at a concentration of 2m*M* SO_4^{2-}. Therefore, 5 m*M* SO_4^{2-} was selected. Addition of methanol to the mobile phase was also effective for the separation of degradation products from PIPC.

Based on the above findings, a mixture of 10 m*M* TBAH and 5 m*M* K_2SO_4 (pH 4.1), acetonitrile and methanol (1000:300:25) was selected for routine assays of I and PIPC.

3.5. Determinations of I and PIPC in pharmaceutical preparations

The mass ratio of I and PIPC in pharmaceutical preparations was 1:4, whereas their peak-area ratio was 1:8 at 220 nm under the HPLC conditions described above. Detection of

I and PIPC was investigated using the wavelength-scanning technique for the assay of low concentrations of I. The repeatabilities of the assays of I and PIPC detection at 220 nm and with wavelength-scanning detection (I at 220 nm and PIPC at 270 nm) are shown in Table 4, where the wavelength was scanned from 220 to 270 nm 8 min after injection. This result revealed that the repeatabilities based on wavelength-scanning detection are better than those based on detection at 220 nm. Hence wavelength-scanning detection was obviously useful for assays of I and PIPC in pharmaceutical preparations and was selected in order to obtain higher repeatabilities. A typical chromatogram of standard I and PIPC is shown in Fig. 2, where methyl benzoate was used as an internal standard.

3.6. Linearity and precision

The linearity of the response was good for both I and PIPC throughout the range of concentrations studied (I, 0–7.5 μg per 10 μl; PIPC, 0–30 μg per 10 μl). Regression analysis of mass (x) *versus* peak-area ratio of I and PIPC to the internal standard (y) gave straight lines with correlation coefficients of 1.000 and 0.9998 ($y = -0.003 + 0.1153x$, $y = -0.019 + 0.0585x$), respectively. The relative standard deviations (R.S.D.) for I and PIPC for six or seven replicates assays were 0.45% and 0.33% respectively, for within-day assay, and 0.78% and 0.52%, respectively for day-to-day assay.

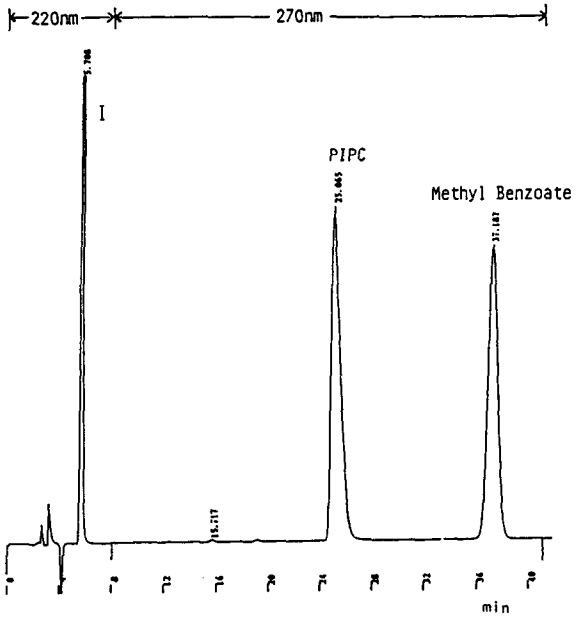


Fig. 2. Chromatogram of standard in the determination of pharmaceutical preparations. Chromatographic conditions: column, Ultron ODS-X; mobile phase, mixture of 10 m*M* tetrabutylammonium hydroxide (TBAH) and 5 m*M* K_2SO_4 (pH 4.1, adjusted with phosphoric acid), acetonitrile and methanol (1000:300:25); column temperature, room temperature; flow-rate, 0.7 ml/min; detection, I at 220 nm, PIPC at 270 nm; sample size, 25 μg.

3.7. Application of the proposed method

The stability of the solution of a sample of the combined formulation of I and PIPC at 5°C was investigated by the proposed method. After

Table 4
Repeatabilities of assays of I and PIPC with detection at 220 nm or with wavelength scanning from 220 to 270 nm

Parameter	I		PIPC	
	220 nm	220–270 nm[a]	220 nm	220–270 nm[a]
Mean (%)[b,c]	100.4	99.9	100.0	99.8
R.S.D. (%)[c]	0.50	0.27	0.54	0.15

Chromatographic conditions as in Fig. 2.
[a] The wavelength was scanned 8 min after injection.
[b] Percentage to the labelled amounts of I and PIPC in pharmaceutical preparations.
[c] $n = 6$.

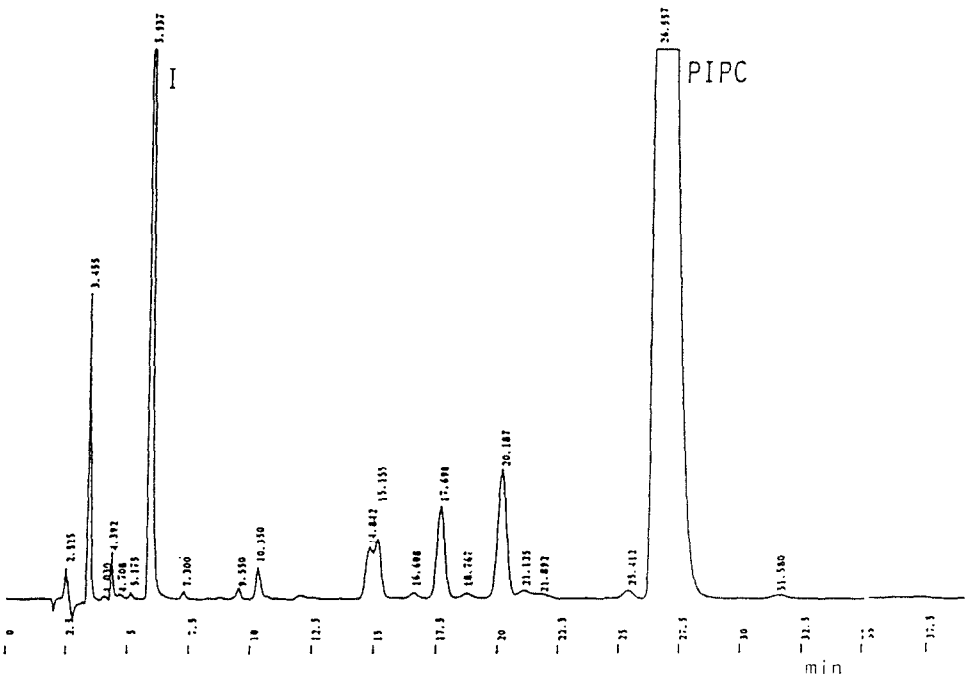


Fig. 3. Chromatogram of skin test ampoule. An ampoule was stored at 40°C for 3 months was used. The sample was treated as described under Experimental. Chromatographic conditions as in Fig. 2, except for detection wavelength, 220 nm.

storage for 36 h at 5°C, the residual contents of **I** and PIPC were 99.5% and 101.1%, respectively.

Fig. 3 shows the chromatogram of **I** and PIPC in a skin test ampoule (40°C, 3 months), in the form of a pharmaceutical preparation for allergenic testing. The homogeneities of the **I** and PIPC peaks were verified by using photodiode-array detection. The specific chromatogram of the degraded sample at 220 and 230 nm is shown in Fig. 4. The ratios of the absorbances at 220

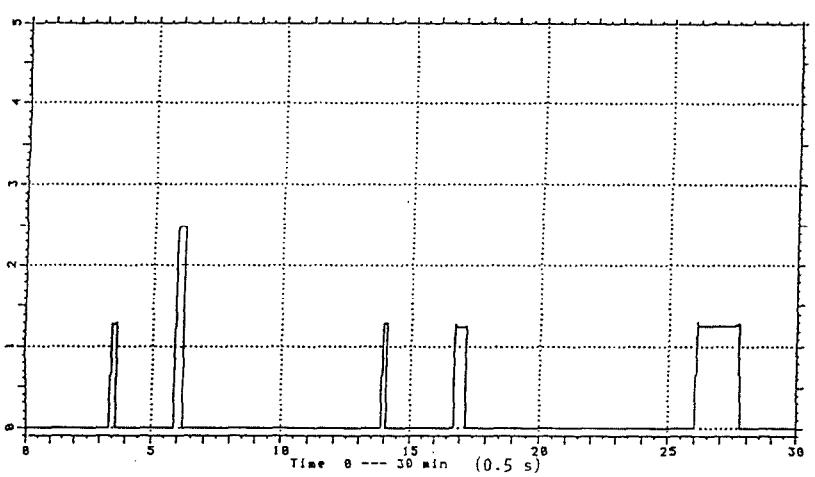


Fig. 4. Specific chromatogram of heat-degraded sample. Sample and chromatographic conditions as in Fig. 3, except for detection wavelength.

and at 230 nm for **I** and PIPC were constant, and a rectangular chromatogram was obtained. These results reveal that **I** and PIPC are well separated from their degradation products, and that none of the degradation products interfere with the assays of **I** and PIPC.

When a sample of the combined formulation of **I** and PIPC was degraded for 3 months at 40°C, the resulting mixtures contained many degradation products. The chromatogram of the degraded sample at 220 nm is shown in Fig. 5. Double the volumes used to obtain the chromatogram in Fig. 3 were injected, but **I** and PIPC were still well separated from their degradation products. Six replicate analyses of the sample showed that the content of degradation products was $16.27 \pm 0.20\%$ (mean ± S.D.), based on the UV absorbance at 220 nm.

In conclusion, the proposed method using wavelength scanning was successfully applied to routine quality control and stability assays of **I** and PIPC. Also, with the slight modification the method will be applicable to the determination of degradation products and the determination of **I** and PIPC in biological fluids.

Acknowledgement

The authors are indebted to Dr. J. Haginaka, Mukogawa Women's University, for helpful comments on the manuscript.

References

[1] P.A. Hunter, K. Coleman, J. Fisher and D. Talor, *J. Antimicrob. Chemother.*, 6 (1980) 455; and references cited therein.

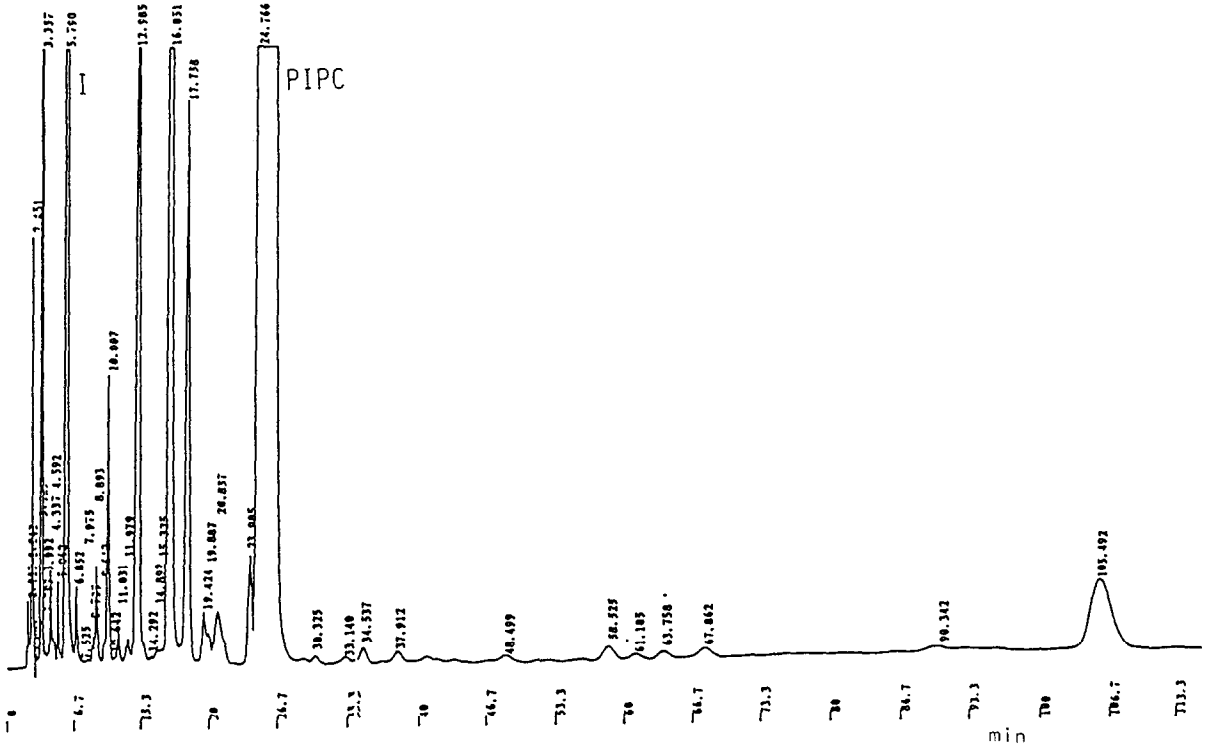


Fig. 5. Chromatogram of heat-degraded sample. Sample and chromatographic conditions as in Fig. 3, except for sample size, 50 μg.

[2] A.R. English, J.A. Retsema and A.E. Girand, J.E. Lynch and W.E. Barth, *Antimicrob. Agents Chemother.*, 14 (1978) 414.
[3] R.G. Micetich, T.W. Hall, S.N. Maiti, P. Sevak, S. Yamabe, N. Ishida, K. Ogawa, M. Tanaka, T. Yamasaki and A. Nakai, in J. Ishigami (Editor), *Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985*, University of Tokyo Press, Tokyo, 1985, pp. 249–250.
[4] S. Aronoff, P. Labrozzi and S. Yamabe, in J. Ishigami (Editor), *Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985*, University of Tokyo Press, Tokyo, 1985, pp. 1268–1269.
[5] S. Aronoff, P. Labrozzi, M. Jacobs and S. Yamabe, in J. Ishigami (Editor), *Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985*, University of Tokyo Press, Tokyo, 1985, pp. 1270–1271.
[6] F. Moosdeen, J.D. Williams and S. Yamabe, in J. Ishigami (Editor), *Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985*, University of Tokyo Press, Tokyo, 1985, pp. 1272–1273.
[7] N. Ishida, A. Hyodo, C. Hanehara, M. Miyake, Y. Kawaguchi and S. Yamabe, in J. Ishigami (Editor), *Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985*, University of Tokyo Press, Tokyo, 1985, pp. 1274–1275.
[8] M.D. Kitzis, L. Gutmann, S. Yamabe and J.F. Acar, in J. Ishigami (Editor), *Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985*, University of Tokyo Press, Tokyo, 1985, pp. 1276–1277.
[9] M.R. Jacobs, S.C. Aronoff, S. Johenning and S. Yamabe, in J. Ishigami (Editor), *Proceedings of the 14th International Congress of Chemotherapy, Kyoto, 1985*, University of Tokyo Press, Tokyo, 1985, pp. 1278–1279.
[10] S.C. Aronoff, M.R. Jacobs, S. Johenning and S. Yamabe, *Antimicrob. Agents Chemother.*, 26 (1984) 580.
[11] P.C. Appelbaum, M.R. Jacobs, S.K. Spangler and S. Yamabe, *Antimicrob. Agents Chemother.*, 30 (1986) 789.
[12] L. Gutmann, M.D. Kitzis, S. Yamabe and J.F. Acar, *Antimicrob. Agents Chemother.*, 29 (1986) 955.
[13] F. Higashitani, A. Hyodo, N. Ishida, M. Inoue and S. Mitsuhashi, *J. Antimicrob. Chemother.*, 25 (1990) 567.
[14] T. Marunaka, E. Matsushima, Y. Minami, K. Yoshida and R. Azuma, *Chem. Pharm. Bull.*, 36 (1988) 4478.
[15] E. Matsushima, K. Yoshida, R. Azuma, Y. Minami and T. Marunaka, *Chem. Pharm. Bull.*, 36 (1988) 4593.
[16] I. Saikawa, S. Takano, C. Yoshida, E. Saitoh, T. Mochida, H. Sakai, H. Takashita, F. Yamamoto and Y. Sugimoto, *Yakugaku Zasshi*, 97 (1977) 995.
[17] *The Parmacopoeia of Japan*, The Society of the Japanese Pharmacopoeia, Tokyo, 12th ed., 1991, p. 36.