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Short communication

High-performance liquid chromatographic analysis of cyclosporin A and its oral solutions

Aleš Husek

Galena Co., Department of Analytical Chemistry, R and D, Ostravská 29, 747 70 Opava-Komárov, Czech Republic

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Abstract

Different columns and conditions were evaluated for the HPLC analysis of cyclosporin A, its congeners and degradation products. The optimised conditions were compared with the pharmacopoeial HPLC method for cyclosporin A analysis and some modifications, applicable among others for the stability studies of oral dosage forms, are proposed.

Keywords: Pharmaceutical analysis; Cyclosporins

1. Introduction

Cyclosporin A (Fig. 1) is a cyclic undecapeptide produced by some imperfect fungi together with a number of its congeners differing by 1–3 amino acids [1]. Nowadays it is widely used to prevent transplanted organ rejection and for the treatment of various autoimmune diseases (Consupren, Galena;

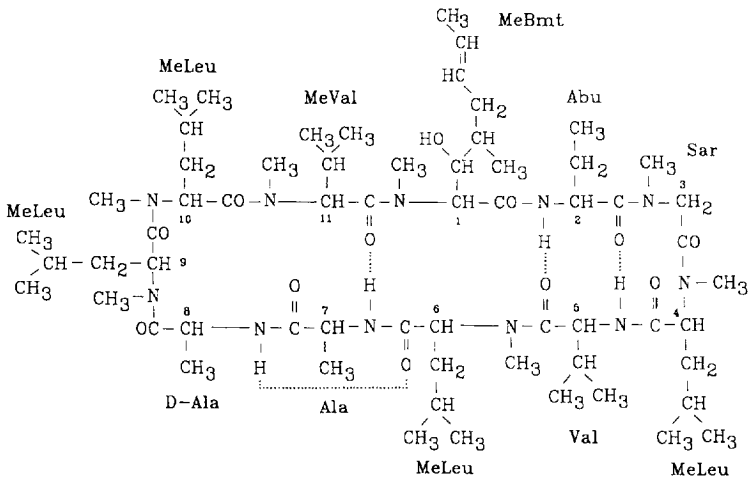

Fig. 1. Cyclosporin A: Cyclo[[(*E*)-(2*S*,3*R*,4*R*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-N-methylglycyl-N-methyl-L-leucyl-L-valyl-N-methyl-L-leucyl-L-alanyl-D-alanyl-N-methyl-L-leucyl-N-methyl-L-leucyl-N-methyl-L-valyl].

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Implanta, Hanmi Pharmaceutical Co.; Sandimmun, Sandoz). With regard to the current rules (e.g., US Food and Drugs Administration), cyclosporin A is produced with a quality >98.5% [2]. In this respect, one should consider that 1% of minor cyclosporins are tolerated in cyclosporin A and that this amount might be expected no matter who produced the cyclosporin A. This is particularly interesting in view of recent results indicating that industrial fungal strains also produced cyclosporins which were previously detected among human trace metabolites ([Leu^4]Cs=AM4N, [Leu^9]Cs=AM9N) [3–5]. Due to its cyclic structure and lipophilic character, cyclosporin A is very stable under a wide range of experimental conditions; however, its stability is not unlimited. Recently, several degradation pathways have been described: racemisation of [MeVal^{11}] leading to cyclosporin H [6], N–O peptidyl shift providing isocyclosporin A [7–10], acid and Lewis-acid catalyzed opening of the cyclic skeleton [11], singlet oxygen oxidation [12], dehydration, and even the loss of the [MeBmt^1] side chain [13]. Since all derivatives arising from these techniques are non-immunosuppressive, sophisticated analytical methods for the continuous monitoring of the source substance, dosage forms and their stability are necessary. The pharmacopoeial methods used for purity control of cyclosporin A in Europe [14] and in the USA [2] are essentially the same; their description, however, is not sufficiently detailed for some points. In this paper some important details are stressed, mainly the sorbent selection and column temperature control, and a modification of the pharmacopoeial method is proposed, which is advantageous with respect to analysis of cyclosporin A degradation products, especially in oral dosage forms.

2. Experimental

2.1. Reagents

Water was drawn from an Ultrapur water system (Goro s.r.o., Prague, Czech Republic). HPLC-grade acetonitrile was obtained from Riedel de-Haen (Seelze, Germany). *tert.*-Butyl methyl ether for HPLC and sodium dodecyl sulfate LAB were purchased from Merck (Darmstadt, Germany). Sodium hydroxide and phosphoric acid (both analytical-reagent grade) were obtained from Lachema (Brno, Czech Republic). All cyclosporin standards met the Galena internal standards. The used oral dosage forms were: Consupren lot 201195 (Galena, Czech republic), Implanta 100 mg lot 136004 (Hanmi Pharmaceutical Co., South Korea), Sandimmun lot 061 0693 and Neoral lot 002MFD1193 (both Sandoz, Switzerland). The used lot of cyclosporin A substance: Galena lot 301095.

2.2. Chromatographic conditions

A Thermo Separation Products (Fremont, CA, USA) modular chromatograph consisted of a solvent-delivery system Model P1000, an autosampler Model AS1000 with 20-μl loop, a variable-wavelength UV detector Model UV1000 with the wavelength set at 210 nm and a controller Model SN4000. Data collection and reporting was by a PC1000 data acquisition system. The column temperature was maintained at 80°C by a Techlab Model T-1 column thermostat (Erkerode, Germany). The thermostat was filled with glycerine unless indicated otherwise. A 1 m×0.25 mm I.D. stainless-steel capillary was placed into the thermostat before the column. Used columns (sorbent, column dimension, particle size, manufacturer): Bakerbond NP Octadecyl, 250×4.6 mm I.D., 5 μm, Baker (Philipsburg, NJ, USA); Hypersil ODS, 250×4.6 mm I.D., 3 μm, Grom (Herrenberg, Germany); Kromasil 100 C_{18}, 250×4.6 mm I.D., 5 μm, Grom; LiChrosorb RP-18, 250×4 mm I.D., 5 μm, Merck; LiChrospher 100 RP-18, 250×4 mm I.D., 5 μm, Merck; Nova-Pak C_{18}, 300×3.9 mm I.D., 4 μm, Waters (Milford, MA, USA); Nucleosil 100 C_{18}, 250×4.6 mm I.D., 5 μm, Grom; Nucleosil 120 C_{18}, 250×4.6 mm I.D., 5 μm, Grom; Nucleosil 300 C_{18}, 250×4 mm I.D., 5 μm, Grom; Zorbax ODS, 250×4.6 mm I.D., 5 μm, Grom.

The pharmacopoeial mobile phase [2,14] was acetonitrile–water–*tert.*-butyl methyl ether–phosphoric acid (430:520:50:1, v/v). The modified mobile phase used for comparative analyses of the test mixture on the individual columns was acetonitrile–water–*tert.*-butyl methyl ether (430:520:50, v/v), with the addition of phosphoric acid (0.02 mol/l) and sodium dodecyl sulfate (0.01 mol/l); the pH was adjusted with concentrated NaOH solution to 3.5, 5.0

or 6.5, respectively. The modified mobile phase used for analyses of the individual oral dosage forms on Hypersil ODS was acetonitrile–water–*tert.*-butyl methyl ether (340:590:72, v/v), with addition of phosphoric acid (0.02 mol/l) and sodium dodecyl sulfate (0.01 mol/l); the pH was adjusted with concentrated NaOH solution to 2.80. All versions of the modified mobile phase were prepared as follows: the calculated amount of sodium dodecyl sulfate was dissolved in the mixture of acetonitrile and water and the solution was filtered using a 0.45-μm membrane filter. After that phosphoric acid and *tert.*-butyl methyl ether were added and the pH was adjusted. The mobile phase was recycled using Jour Research (Onsala, Sweden) solvent-saver Model 1704. The flow-rate was set to 1–2.5 ml/min, so that the retention of cyclosporin A was about 23–33 min. Test mixture of cyclosporin standards was obtained using cyclosporins [Leu^4]Cs, C, B, L, U, A, H, [dihydroMeBmt^1]Cs, G, D, F and isocyclosporin A. The standards were dissolved in a mixture of acetonitrile–water (1:1, v/v), concentration of cyclosporin A was 1 mg/ml and of all others 0.01 mg/ml each.

3. Results and discussion

Ten different sorbents corresponding to USP23 specification L1 were tested: Bakerbond NP Octadecyl, 5 μm; Hypersil ODS, 3 μm; Kromasil 100 C_{18}, 5 μm; LiChrosorb RP-18, 5 μm; LiChrospher 100 RP-18, 5 μm; Nova-Pak C_{18}, 4 μm; Nucleosil 100 C_{18} 5 μm; Nucleosil 120 C_{18} 5 μm; Nucleosil 300 C_{18} 5 μm and Zorbax ODS, 5 μm. Specific surface area, pore size and carbon content of the mentioned sorbents are summarized in Table 1. Analyses of the cyclosporin test mixture containing the common related substances and degradation products were performed on columns filled with these sorbents using original pharmacopoeial mobile phase [2,14] and the modified one at two or three pHs (Table 1). The sorbents were evaluated with respect to their applicability for determination of the principal degradation products of cyclosporin A, i.e., isocyclosporin A and cyclosporin H. With exception of Nucleosil 100 and 300, the resolution of cyclosporin H and cyclosporin A was satisfactory in both pharmacopoeial and modified mobile phases and could be improved by changing to sorbents with lower particle diameters. However, due to tailing of the main peak, complete resolution of both cyclosporins was not achieved on any column.

Using the pharmacopoeial mobile phase isocyclosporin A was not eluted on Bakerbond, Hypersil, Nova-Pak and Zorbax or was coeluted with some of the related substances on LiChrosorb, LiChrospher and Nucleosil 300. Only Nucleosil 100 and 120 and Kromasil could be used for this analysis, however, Nucleosil 100 and Kromasil seem to be less suitable due to the low capacity factor of isocyclosporin A. The retention of isocyclosporin A, the only basic cyclosporine, could be affected under these conditions by ion-exchange interaction with trace metal ions present in silica gel and therefore may depend on silica gel purity. No correlation between specific surface area or carbon content of the sorbents and the isocyclosporin A behaviour was observed. In the case of Nucleosil the influence of sorbent porosity was investigated: retention of isocyclosporin A increased with increasing pore size, its capacity factor on Nucleosil 100 was too low and on the contrary coelution with cyclosporins B and L was observed on Nucleosil 300.

Using the modified mobile phase isocyclosporin A formed an ion-pair with sodium dodecyl sulfate and its retention was therefore pH-dependent. It was eluted in the region between cyclosporins H and D using most of the sorbents investigated and its retention was affected only slightly by the pH. It was proved on Kromasil, Nova-Pak and Nucleosil 120 that no satisfactory resolution of isocyclosporin A from cyclosporin D can be achieved in such cases by the increase of the pH up to 6.5. Only on Bakerbond and Hypersil the retention of isocyclosporin A was strongly increased with a decrease in pH, which is advantageous for analysis of cyclosporin A substance and particularly of its dosage forms.

Relatively high temperature is necessary for analysis of cyclosporin to ensure fast equilibration of individual conformers and therefore to achieve satisfactory peak shape [15–17]. The common air-bath thermostats were not suitable for cyclosporin analysis owing to insufficient heat transfer onto the column. It is advisable to use such thermostat models, which could be filled with a liquid media

Table 1
Sorbent characteristics, retention times of cyclosporin A and relative retention times of some minor cyclosporins[a]

Sorbent	Surface area [m^2/g]	Pore size [Å]	Carbon [%]	Flow-rate [ml/min]	pH[b]	CsC	CsB	CsL	CsU	CsA [min]	CsH	A/H resoln.	H_2A[c]	CsG	CsD	CsF	isoA[d]
Bakerbond	170	120	12	1.5	USP23	0.66	0.79	0.86	0.95	27.7	1.09	1.45	1.25	1.31	1.39	1.88	n.d.
				1.5	3.5	0.67	0.79	0.86	0.96	25.2	1.09	1.48	1.25	1.31	1.39	**1.86**	1.82
				1.5	5.0	0.67	0.79	0.86	0.96	25.1	1.09	1.57	1.25	1.31	1.39	1.86	1.50
Hypersil	175	120	9.5	1.5	USP23	0.67	0.79	0.85	0.94	33.0	1.07	1.63	1.24	1.32	1.39	1.88	n.d.
				1.5	3.5	0.68	0.79	0.86	0.94	29.3	1.08	1.50	1.24	1.31	1.39	**1.86**	1.86
				1.5	5.0	0.67	0.79	0.85	0.94	29.5	1.08	1.58	1.24	1.31	1.38	1.86	1.51
Kromasil	340	100	19	2.5	USP23	0.61	0.75	0.83	0.93	30.3	1.11	1.30	1.24	1.36	1.44	1.98	0.12
				2.5	3.5	0.61	0.75	0.83	0.94	30.6	**1.12**	1.48	1.25	1.36	1.45	1.98	1.17
				2.5	5.0	0.61	0.75	0.83	0.94	30.6	1.12	1.50	1.24	**1.36**	**1.44**	1.98	1.41
				2.5	6.5	0.61	0.75	0.83	0.94	30.9	1.12	1.58	1.25	1.36	**1.45**	1.97	1.46
LiChrosorb	300	100	11.4	2	USP23	0.63	0.77	0.85	0.92	26.6	1.06	1.03	1.22	**1.33**	**1.40**	1.86	1.35
				2	3.5	0.63	0.77	0.85	0.92	26.4	1.08	0.99	1.22	1.33	**1.41**	1.86	1.41
				2	5.0	0.62	0.77	0.85	0.92	27.4	1.08	1.06	1.22	**1.33**	1.41	1.86	1.34
LiChrospher	350	100	21	2	USP23	0.62	0.76	0.84	0.93	29.1	1.11	1.46	1.24	1.35	1.43	**1.93**	1.94
				2	3.5	0.63	0.76	0.84	0.93	27.9	1.11	1.53	1.23	1.34	**1.43**	1.92	1.41
				2	5.0	0.63	0.76	0.84	0.94	27.9	1.11	1.62	1.23	1.34	**1.43**	1.91	1.43
Nova-Pak	120	60	7	1.5	USP23	0.62	0.76	0.84	0.94	25.2	1.14	2.27	1.25	1.37	1.46	1.96	n.d.
				1.5	3.5	0.63	0.76	0.85	0.94	22.8	1.14	2.16	1.25	**1.36**	1.45	1.96	1.34
				1.5	5.0	0.63	0.77	0.85	0.94	22.7	1.14	2.18	1.25	1.36	**1.46**	1.96	1.45
				1.5	6.5	0.63	0.77	0.85	0.94	22.8	1.14	2.27	1.24	1.36	**1.45**	1.97	1.48
Nucleosil 100	350	100	14	2.2	USP23	0.63	0.77	0.85	0.92	27.5	1.07	fused	1.24	1.32	1.40	1.82	0.23
Nucleosil 120	200	120	11	2	USP23	0.62	0.76	0.84	0.92	30.4	1.08	1.07	1.24	1.33	1.41	1.86	0.49
				2	3.5	0.63	0.76	0.85	0.92	29.5	**1.08**	1.02	1.24	1.33	1.41	1.85	1.12
				2	5.0	0.63	0.76	0.85	0.92	29.1	1.08	1.04	1.24	**1.33**	**1.41**	1.85	1.37
				2	6.5	0.63	0.77	0.85	0.93	29.9	1.08	1.06	1.24	1.33	**1.41**	1.86	1.37
Nucleosil 300	100	300	6	1	USP23	0.70	**0.81**	**0.86**	0.94	23.1	1.05	fused	1.21	1.29	1.35	1.80	0.83
Zorbax	300	70	20	2	USP23	0.60	0.74	0.83	0.93	25.2	1.14	1.09	1.24	1.38	1.47	1.98	n.d.
				2	3.5	0.60	0.74	0.83	0.92	27.2	1.12	0.97	1.26	1.38	**1.48**	2.02	1.54
				2	5.0	0.60	0.74	0.83	0.92	28.0	1.13	1.04	1.26	1.38	**1.48**	2.02	1.48

[a] The bold printed values mark the cyclosporins coeluted with isocyclosporin A.
[b] pH value of the modified mobile phase; "USP23" indicates the analyses in pharmacopeial mobile phase.
[c] [dihydroMeBmt[1]]Cs.
[d] n.d.—isocyclosporin A (isoA) was not eluted out of the column.

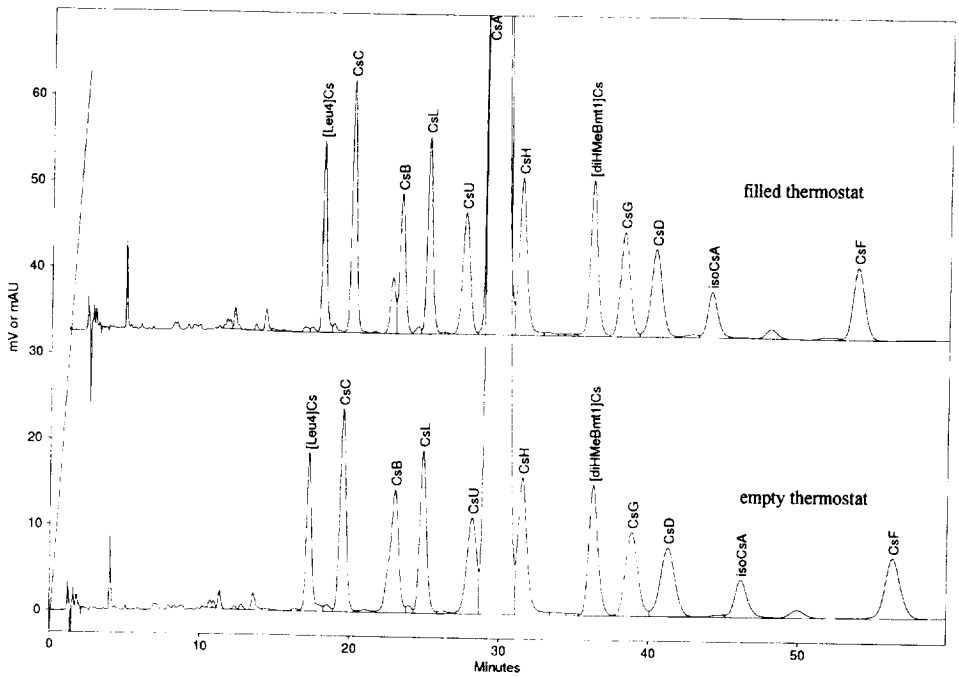

Fig. 2. Influence of thermostat filling on the column efficiency. Conditions: Hypersil ODS, 250×4.6 mm I.D., 3 μm, at 80°C; acetonitrile–water–*tert.*-butyl methyl ether (430:520:50, v/v), with addition of phosphoric acid (0.02 mol/l) and sodium dodecyl sulfate (0.01 mol/l); the pH value was adjusted with concentrated NaOH solution to 5.0, the flow-rate was 1.7 ml/min. The thermostat was empty or filled with glycerine, respectively.

(e.g., glycerine). This effect is illustrated on Fig. 2, where two chromatograms of the cyclosporin test mixture analysed on Hypersil ODS are depicted. The first analysis was performed on the column placed in the empty thermostat, subsequently the thermostat was filled with glycerine and the second analysis was done. The resolution of cyclosporin U and cyclosporin A which is a part of system suitability test in the pharmacopoeial method was in the filled thermostat $R_{UA}>1$, while in the empty thermostat partial fusion of both peaks appeared. The number of theoretical plates (per meter) for the main peak was increased from 30 200 in the empty thermostat to 40 700 with the column submersed into glycerine.

As mentioned above, the modified pharmacopoeial method seems to be advantageous for stability tests of cyclosporin A and of its dosage forms. Using the suitable sorbent (Bakerbond or Hypersil) it is possible to vary the elution of isocyclosporin A in a wide range of retention times by adjusting the pH of the mobile phase. It means, that a pH can be chosen to achieve complete resolution of isocyclosporin A from all peaks of related substances or placebo. Retention of cyclosporin H is influenced by mobile phase composition too, namely by small changes of *tert.*-butyl methyl ether content (total content 5–8%, v/v). The virtue of the modification was demonstrated on analysis of cyclosporin A substance and of various oral dosage forms (Consupren, Implanta, Sandimmun and Neoral). The substance was analysed using the mobile phase of the same solvent relation compared to the pharmacopoeial method (Fig. 3). Under these conditions cyclosporin H would be eluted closely after the main peak before the peak of CsT. The pH was set to 5.0 so that isocyclosporin A was eluted in the free region between cyclosporins D and F. However, by analysis of the oral dosage forms the mentioned regions were taken by peaks originated from placebo. Therefore it was necessary to increase the *tert.*-butyl methyl ether content, in order to increase retention of cyclosporin H. The pH of the mobile phase had to be decreased to 2.80 simultaneously, to shift isocyclosporin A into the free region after the 80th minute. The method

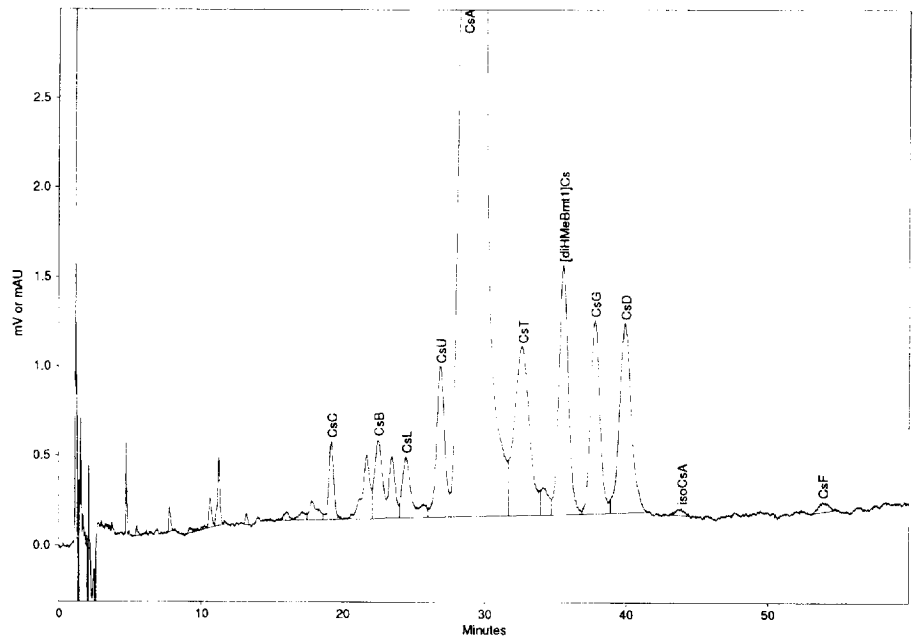

Fig. 3. Analysis of cyclosporin A substance (Galena lot 301095) in the modified mobile phase. Conditions: Hypersil ODS, 250×4.6 mm I.D., 3 μm, at 80°C; acetonitrile–water–*tert.*-butyl methyl ether (430:520:50, v/v), with addition of phosphoric acid (0.02 mol/l) and sodium dodecyl sulfate (0.01 mol/l); the pH value was adjusted with concentrated NaOH solution to 5.0, the flow-rate was 1.7 ml/min.

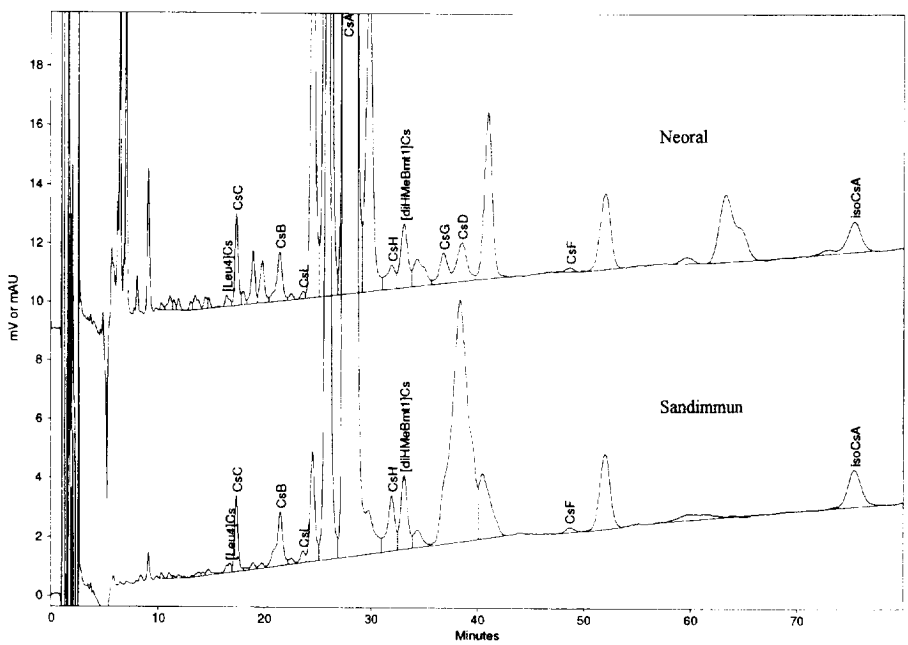

Fig. 4. Analyses of cyclosporin A oral dosage forms (Sandimmun and Neoral) in the modified mobile phase. Conditions: Hypersil ODS, 250×4.6 mm I.D., 3 μm, at 80°C; acetonitrile–water–*tert.*-butyl methyl ether (340:590:72, v/v), with addition of phosphoric acid (0.02 mol/l) and sodium dodecyl sulfate (0.01 mol/l); the pH value was adjusted with concentrated NaOH solution to 2.80, the flow-rate was 2 ml/min.

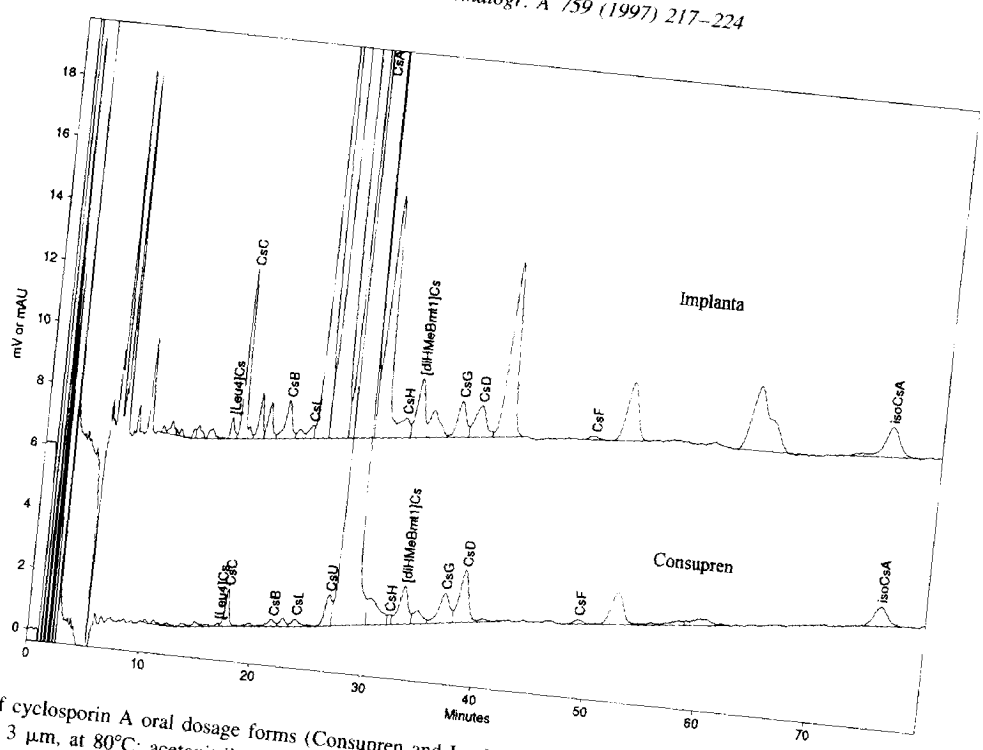

Fig. 5. Analyses of cyclosporin A oral dosage forms (Consupren and Implanta) in the modified mobile phase. Conditions: Hypersil ODS, 250×4.6 mm I.D., 3 μm, at 80°C; acetonitrile–water–*tert.*-butyl methyl ether (340:590:72, v/v), with addition of phosphoric acid (0.02 mol/l) and sodium dodecyl sulfate (0.01 mol/l); the pH value was adjusted with concentrated NaOH solution to 2.80, the flow-rate was 2 ml/min.

was then applicable on the all mentioned cyclosporin oral dosage forms (Figs. 4 and 5). The combination of high column temperature and relatively low pH of the mobile phase causes sorbent hydrolysis, mobile phase recycling results subsequently in moderate baseline drift.

Using a column filled with Hypersil ODS the modified pharmacopoeial method was validated and compared with the original method. Statistically significant differences were not found either in accuracy or in the precision of cyclosporin A determination.

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