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Resolution of several racemic 3-hydroxy-1,4-benzodiazepin-2-ones by high-performance liquid chromatography on a chiral silica-bonded stationary phase*

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Abstract: The resolution of four racemic 3-hydroxy-1,4-benzodiazepin-2-ones, widely used in therapeutics, by means of a chiral stationary phase is described. The chiral selector used is (*S*)-*N*-(3,5-dinitrobenzoyl)phenylalanine. This chiral stationary phase showed both good enantioselectivity and efficiency for the compounds. Elution times were in all cases shorter than those previously reported for such compounds on different stationary phases. Racemic oxazepam was used to evaluate the loading capacity of the chiral stationary phase.

Keywords: *Chiral stationary phase; benzodiazepines; resolution of enantiomers; high-performance liquid chromatography.*

Introduction

Drugs having the structure of 1,4-benzodiazepine are extensively used in therapeutics either as anxiolytic or as hypnotic agents. Several 1,4-benzodiazepines are chiral due to the presence of a 3-hydroxy group in the diazepine ring. 3-Hydroxy-1,4-benzodiazepines are particularly important because non-chiral benzodiazepines are usually metabolized by hydroxylation in the 3 position of the diazepine ring originating pharmacologically active metabolites. Moreover, the pharmacological activity of such benzodiazepines has been shown to be enantiodependent [1].

Among the 3-hydroxy-1,4-benzodiazepin-2-ones, oxazepam and lorazepam constitute 30% of the market of anxiolytic drugs in France.

Although 3-hydroxybenzodiazepines are frequently used as test compounds to evaluate new chiral stationary phases (CSP) or optimize chromatographic methods, chromatographic resolution remains limited to one or two of them in most cases. In such studies oxazepam is the most commonly used 3-hydroxybenzodiazepine, whereas temazepam and lormetazepam are of very limited use. To date, the chromatographic resolution of all four benzodiazepines, i.e. oxazepam, temazepam, lorazepam and lormetazepam, has only been described in one report [2].

Previous experiments have involved the use of almost all available chiral stationary phases. Thus oxazepam is resolved by bonded-silica gel phases bearing various chiral selectors: (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine (DNBPG), (*S*)-*N*-(3,5-dinitrobenzoyl)leucine (DNBL) [3, 4], (*S*)-*N*-(3,5-dinitrobenzoyl)tyrosine-*n*-butylamide (DNBTyr) [5] or (*S,S*)-*N*-(3,5-dinitrobenzoyl)-1,2-diphenylethane-1,2-diamine (DNB-DPEDA) [2]. This analyte is also resolved by poly-*N*-acryloylamino acid derivatives [4, 6–9] coated or non-coated onto silica gel (preparative separation [10]), β-cyclodextrin (β-CD) [11, 12], (*R*)- and (*S*)-naphthylethylcarbamate substituted β-cyclodextrin (NEA-β-CD) phases [13], cellulose trisphenylcarbamate and tris-4-methylbenzoate [14], phases whose chiral selectors are cinchona alkaloids [15, 16], proteic phases such as bovine serum albumin (BSA) [17–22], α_1-acid glycoprotein (α_1-AGP) [23] and human serum albumin (HSA) [24]. Resolution of temazepam is achieved by using DNB-DPEDA [2], *N*-formylphenylalanine [8], HSA [24], BSA [22], cellulose triacetate (CTA) [12] and NEA-β-

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CD [13] phases. Enantiomers of lorazepam are separated on DNB-DPEDA [2], DNB-PG [3, 25], *N*-3,5-dinitrophenylaminocarbonyl-(*S*)-valine [25], poly-*N*-acryloyl-(*S*)-phenylalanine ethyl ester [7, 25], NEA-β-CD [13], cinchona-alkaloid containing phases [15, 16], cellulose trisphenylcarbamate [25], α_1-AGP and ovomucoid protein (OVM) [26], BSA [20] and HSA [24] phases. Chiral separation of lormetazepam is obtained only on DNB-DPEDA [2, 27], cinchona-alkaloid bonded phases [15] and BSA [20].

Results obtained from all these studies depend on both the racemic benzodiazepine and the CSP used. Thus, elution time fluctuates according to the chiral selector, ranging from 10 to 60 min. Usually the 3-hydroxybenzodiazepines are strongly retained by stationary phases, producing wide peaks and large Height Equivalent of a Theoretical Plate (HETP) values for the last enantiomer eluted [17, 19, 23, 24, 26].

Recently, a new chiral stationary phase was obtained by condensation of a chiral silane, bearing an (*S*)-*N*-(3,5-dinitrobenzoyl)phenylalanine group as a chiral selector, on silica gel. The condensation step was followed by deactivation of remaining silanol groups by drastic end-capping treatment with hexamethyldisilazane. One of the special features of this CSP is to exhibit shorter elution times than those shown by CSP bearing the same chiral selector but prepared from a γ-aminopropylsilica gel, or those obtained by condensation of the chiral silane on the silica gel but without the subsequent end-capping treatment [28].

This study describes the resolution of four 3-hydroxybenzodiazepines (Fig. 1) on this CSP. In order to assess the influence of the origin of silica gel on the resolution, two CSP prepared in identical way from Nucleosil 100-5 (CSP-1) or Spherisorb S5W (CSP-2) were tested (Fig. 2). The CSP exhibiting the best benzodiazepine resolution was selected, in order to study its loading capacity by injecting different amounts of racemic oxazepam.

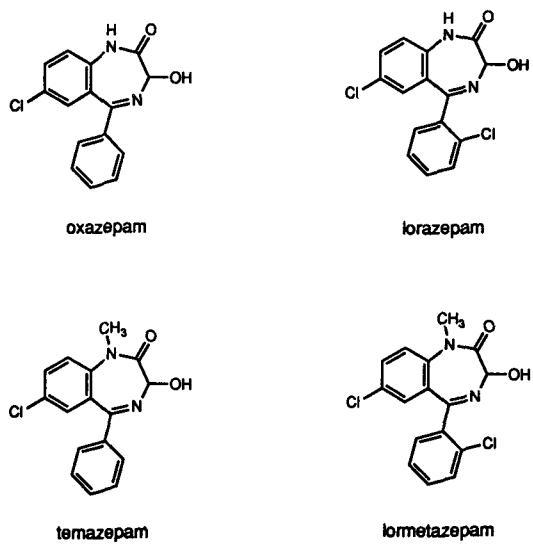

Figure 1
Racemic 3-hydroxybenzodiazepines.

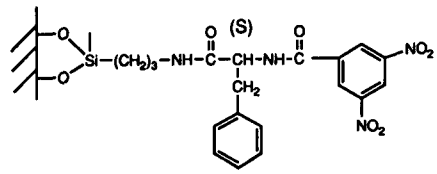

Figure 2
Structure of the chiral selector of CSP.

Experimental

Apparatus

The chromatographic system consisted of a Constametric II pump (LDC, Division of Milton Roy Company, Riviera Beach, FL, USA), a Valco UHP-7K loop automatic injector (Valco, Houston Instruments Co., TX, USA), with a loop volume of 25 μl, a Spectromonitor III UV detector (LDC, Analytical, France), an ICAP 10 calculator-integrator (Delsi, Suresnes, France) and a Servotrace monochannel recorder (Sefram, France). UV absorption of the eluent was monitored at 254 nm.

To evaluate the loading capacity of CSP-1, when the injected amount exceeded 0.8 μg, a 250 μl sample loop was fitted to the injector and the detector wavelength was shifted to 290 nm.

Columns

Two CSP bearing the same chiral selector were used. They were obtained by condensation of *N*-(*N*-(3,5-dinitrobenzoyl)-(*S*)-phenylalanyl)-3-triethoxysilylpropylamide on two different spherical silica gels, followed by an end-capping treatment with hexamethyldisilazane as described [28]. CSP-1 was obtained from Nucleosil 100-5 (5 μm, 100 Å, Macherey–Nagel) and CSP-2 from Spherisorb S5W (5 μm, 80 Å, Phase Separations Ltd). These

materials were packed into stainless-steel columns (100 × 4.6 mm i.d.) using the slurry technique (methanol, 450–500 atm). Columns packed with CSP-1 are now available from Interchim (Montluçon, France).

The elution time of a non-retained solute (t_0) was measured with 1,3,5-tri-*tert*-butylbenzene. Experiments were carried out at room temperature.

Mobile phases and solutes

Various mixtures of heptane, 2-propanol, methanol and dioxane were used as mobile phases. Solvents, of HPLC grade, were purchased from Prolabo (Paris, France) and Merck (Darmstadt, Germany) and used without further purification. The flow rate was 1 ml min^{-1} in all cases.

Racemic oxazepam, temazepam and lorazepam were kindly provided by Wyeth-France Laboratories (Paris, France). Lormetazepam was a gift from Interchim (Montluçon, France).

Results and Discussion

Results obtained in the resolution of the 3-hydroxybenzodiazepines on CSP-1 and CSP-2 are shown in Table 1. The mixture of heptane–2-propanol–methanol (63:27:10, v/v/v) gave the best results.

Influence of the origin of silica gel on the performance of the CSP

Differences in the physical properties of silica depending on the manufacturer [29] may originate differences in the chromatographic behaviour of the stationary phases prepared from them. In the present case, the surface concentration of surface-bonded chiral entities, calculated from elemental analyses of CSP, is slightly higher in CSP-1 (1.20 μmol m^{-2}) than in CSP-2 (1.01 μmol m^{-2}) [28]. This may be due to the different quantity of residual surface silanol groups on the silica matrix and may be the origin of the better performance of CSP-1 versus CSP-2, as shown in Table 1.

The four 3-hydroxybenzodiazepines were resolved in elution times below 7 min on CSP-1 (Fig. 3). These values are considerably lower than those reported in the literature, even if the length of the column is taken into account. This result confirms the advantage of the new method of CSP preparation.

Influence of the structure of benzodiazepines on resolution

If the resolution of oxazepam and lorazepam, on one hand, and the resolution of temazepam and lormetazepam, on the other, are compared, it is clear that the chiral recognition is better when a hydrogen atom is present on the nitrogen atom at position 1 of

Table 1
Chromatographic parameters for the separation of test-compounds

Compound	Mobile phase*	k'_1†		α‡		Rs§		N_1‖		N_2	
		CSP-1	CSP-2	CSP-1	CSP-2	CSP-1	CSP-2	CSP-1	CSP-2	CSP-1	CSP-2
Oxazepam	A	2.32	3.38	1.26	1.17	1.79	0.83	1799	657	1845	722
	B	2.41	4.09	1.25	1.15	1.47	0.57	1180	456	1429	388
	C	2.58	2.62	1.34	1.19	1.51	0.74	652	610	903	496
Temazepam	A	2.77	3.18	1.13	1.11	1.03	0.73	1961	1343	2344	1246
	B	1.63	2.46	1.18	1.14	0.91	0.72	1096	1024	1154	859
	C	4.41	2.99	1.14	1.14	0.99	0.72	1489	934	1147	730
Lorazepam	A	2.28	3.12	1.57	1.37	3.76	1.75	1755	761	2362	875
	B	2.38	3.91	1.55	1.33	3.30	1.29	1329	481	1959	510
	C	2.68	2.48	1.76	1.36	2.96	1.43	691	711	788	592
Lormetazepam	A	2.62	3.24	1.28	1.23	2.15	1.57	2135	1578	2259	1509
	B	1.59	2.22	1.30	1.24	1.72	1.09	1534	777	1770	839
	C	3.18	3.12	1.34	1.22	2.12	1.08	1336	787	1411	765

*A: heptane–2-propanol–methanol (63:27:10, v/v/v); B: heptane–dioxane–2-propanol (60:30:10, v/v/v); C: heptane–2-propanol (50:50, v/v).

†Capacity factor: $k'_1 = tr_i - t_0/t_0$.

‡Selectivity factor: $\alpha = k'_1/k'_2$.

§Resolution: $RS = 2(tr_2 - tr_1)/(w_2 + w_1)$, where w is the baseline peak width.

‖Number of theoretical plates: $N_i = 16(tr_i/w_i)^2$.

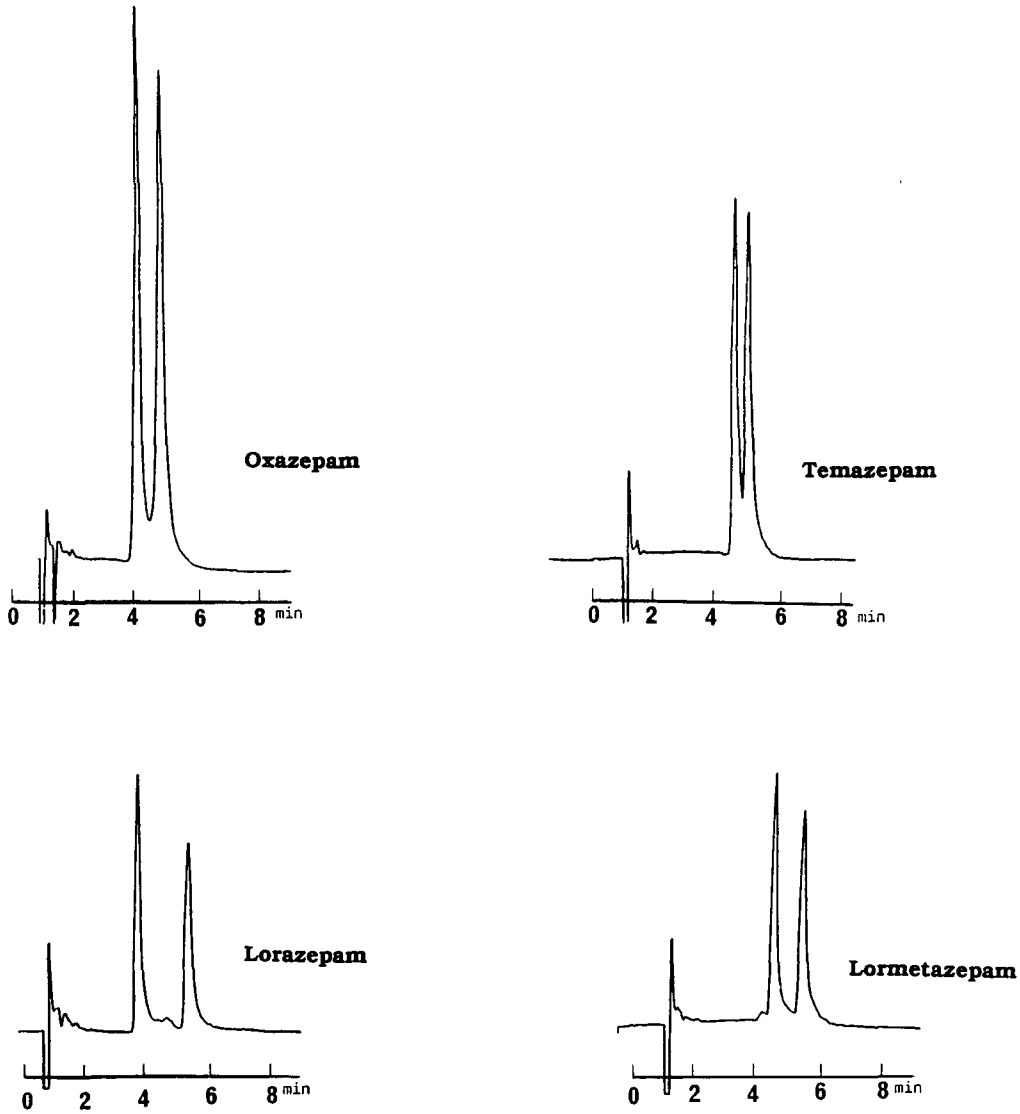

Figure 3
Resolution of 3-hydroxybenzodiazepines on CSP-1. Eluent: heptane–2-propanol–methanol (63:27:10, v/v/v). Flow rate: 1 ml min^{-1}.

the diazepine ring. Therefore, it seems that the hydrogen interaction that can then take place between the NH group in the benzodiazepine, and the carbonyl group in the chiral centre of the chiral selector improves the chiral recognition [3]. In contrast, even when the same interactions can be expected because of the structure of the chiral selector, the opposite is observed on DNB-DPEDA phases. In that case, temazepam and lormetazepam are better resolved than oxazepam and lorazepam [2]. This constitutes an example of the difficulty in the generalization of chiral recognition between solutes and stationary phases.

It can be even noted that lorazepam and lormetazepam are better resolved than their analogous oxazepam and temazepam. The only difference between such compounds is the presence on the former, of a chlorine atom on the phenyl group at position 5 of the diazepine ring. This substituent seems to improve the enantioselectivity of the CSP. This favourable effect on the resolution of 3-hydroxybenzodiazepines (produced by this chlorine atom) is observed in all CSP which can be properly compared [2, 15, 20].

Effect of sample size on HETP values and on sample equivalent retention volume (R)

The capacity of CSP-1 was evaluated by using oxazepam. Results are shown in Fig. 4. This racemic compound was not the best

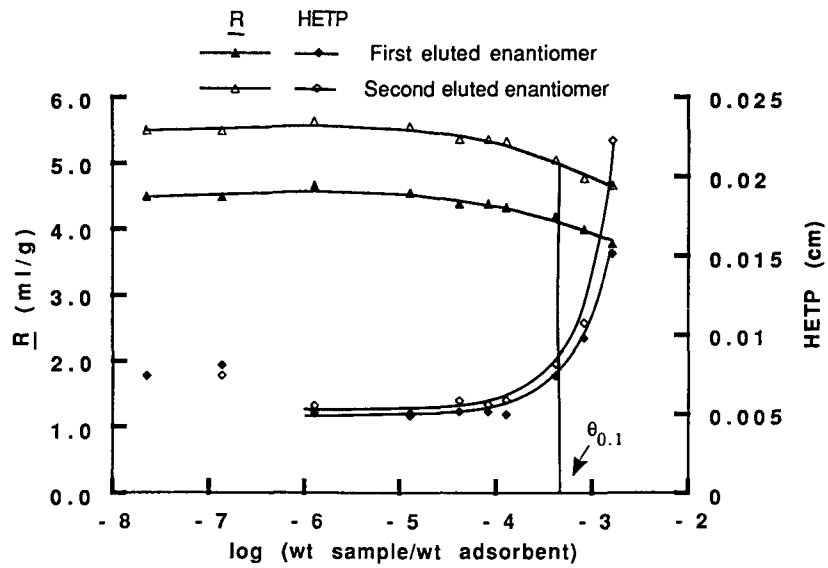

Figure 4
Dependence of HETP and sample retention volume on sample size. R: sample equivalent retention volume (ml g^{-1}) ($R = R' - V°/wt$, where R': sample retention volume (ml), $V°$: column void volume (ml), wt: weight of adsorbent in the column (g)). $R°$: linear isotherm value of R. $\theta_{0.1}$: linear capacity of the column (weight of sample (g) per gram of adsorbent for which R equals 0.9 $R°$) [30].

resolved in the series. However, although lorazepam was better resolved, it could not be used to evaluate the loading capacity of the column because of its low solubility in the mobile phase. In such working conditions the saturation of the column could not be reached.

CSP-1 allows the resolution of the test compound in the linear conditions defined by Snyder [30] up to 4.9×10^{-4} g of racemic oxazepam per gram of adsorbent. This corresponds to 1 μmol of racemic compound in a column packed with 0.6 g of stationary phase. Deviation from linearity of the computed HETP values for the lowest injected amounts of oxazepam (approx. 10 and 80 ng) can probably be attributed to alteration of the experimental conditions (changes in loop volume and wavelength detection).

Additional data can be deduced from results shown in Fig. 4. A parallelism can be observed between curves relating to sample equivalent retention volumes (R) and HETP values for both enantiomers. Therefore, the saturation of the stationary phase is produced by the same mass of solute for both enantiomers. This may indicate either that both enantiomers interact in the same way on the chiral selector of the CSP or, if the first enantiomer eluted is not recognized by the CSP, the distribution coefficient of the more retained enantiomer on the chiral selector is relatively low.

According to our results, the first hypothesis is more likely. Moreover, this idea reinforces the mechanism of chiral recognition in which the formation of diastereomeric complexes between solute and chiral entity on the stationary phase is suggested, as it had already been proposed for this kind of chiral selector [31]. However, the difficulty in representing those complexes in such a way as to explain certain experimental facts already established [32, 33] remains to be solved.

References

[1] H. Möhler and T. Okada, *Science* **198**, 849–851 (1977).
[2] G. Uray and W. Lindner, *Chromatographia* **30**, 323–327 (1990).
[3] W. Pirkle and A. Tsipouras, *J. Chromatogr.* **291**, 291–298 (1984).
[4] S.K. Yang and X. L.-Lu, *J. Pharm. Sci.* **78**, 789–795 (1989).
[5] A. Tambuté, A. Bégos, M. Lienne, P. Macaudière, M. Caude and R. Rosset, *New J. Chem.* **13**, 625–637 (1989).
[6] G. Blaschke, in *Chromatographic Chiral Separations*, Chromatographic Science Series, Vol. 40 (M. Zief and L.J. Crane, Eds), pp. 179–198. Marcel Dekker, New York (1988).
[7] Documentation Merck "Phases chirales", laboratoires Merck-Clevenot, Nogent sur Marne (France).
[8] G. Blaschke, *J. Liq. Chromatogr.* **9**, 341–368 (1986).
[9] D. Arlt, B. Bömer, R. Grosser and W. Lange, *Angew. Chem. Int. Ed. Engl.* **30**, 1662–1664 (1991).
[10] G. Blaschke and H. Markgraf, *Chem. Ber.* **113**, 2031–2035 (1980).

[11] C. Bertucci, E. Domenici, G. Ucello-Barretta and P. Salvadori, *J. Chromatogr.* **506**, 617–625 (1990).
[12] A.F. Fell, T.A.G. Noctor, J.E. Mama and B.J. Clark, *J. Chromatogr.* **434**, 377–384 (1988).
[13] D.W. Armstrong, G.-D. Chang and S.H. Lee, *J. Chromatogr.* **539**, 83–90 (1991).
[14] Application Guide for chiral column selection, Daicel Chemical Industries, Ltd (1989).
[15] C. Rosini, C. Bertucci, D. Pini, P. Altemura and P. Salvadori, *Chromatographia* **24**, 671–676 (1987).
[16] C. Bertucci, C. Rosini, D. Pini and P. Salvadori, *J. Pharm. Biomed. Anal.* **5**, 171–176 (1987).
[17] P. Erlandsson, L. Hansson and R. Isaksson, *J. Chromatogr.* **370**, 475–483 (1986).
[18] P. Erlandsson and S. Nilsson, *J. Chromatogr.* **482**, 35–51 (1989).
[19] S. Andersson, S. Allenmark, P. Erlandsson and S. Nilsson, *J. Chromatogr.* **498**, 81–91 (1990).
[20] S. Andersson, R.A. Thomson and S.G. Allenmark, *J. Chromatogr.* **591**, 65–73 (1992).
[21] S.G. Allenmark, *J. Liq. Chromatogr.* **9**, 425–442 (1986).
[22] S. Allenmark and S. Andersson, *Chiralty* **1**, 154–160 (1989).
[23] A. Walhagen and L.-E. Edholm, *J. Chromatogr.* **473**, 371–379 (1989).
[24] E. Domenici, C. Bertucci, P. Salvadori, G. Félix, I. Cahagne, S. Motellier and I.W. Wainer, *Chromatographia* **29**, 170–176 (1990).
[25] X.-L. Lu and S.K. Yang, *J. Chromatogr.* **535**, 229–238 (1990).
[26] K.M. Kirkland, K.L. Neilson and D.A. McCombs, *J. Chromatogr.* **545**, 43–58 (1991).
[27] W. Lindner, G. Uray and U. Steiner, *J. Chromatogr.* **533**, 373–381 (1991).
[28] L. Oliveros, C. Minguillón, B. Desmazières and P.-L. Desbène, *J. Chromatogr.* **606**, 9–17 (1992).
[29] J. Nawrocki, *Chromatographia* **31**, 1913–205 (1991).
[30] L.R. Snyder, *Anal. Chem.* **39**, 698–704 (1967).
[31] W.H. Pirkle, T.C. Pochapsky, G.S. Mahler, D.E. Corey, D.S. Reno and M. Alessi, *J. Org. Chem.* **51**, 4991–5000 (1986).
[32] L. Oliveros and M. Cazau, *J. Chromatogr.* **409**, 183–192 (1987).
[33] L. Oliveros, C. Minguillón, B. Desmazières and P.-L. Desbène, *J. Chromatogr.* **589**, 53–59 (1992).

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