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High-performance liquid chromatographic separation and measurement of various biogenic compounds possibly involved in the pathomechanism of Parkinson's disease

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Abstract

The work presented here describes an optimised, reversed-phase high-performance liquid chromatographic (RP-HPLC) method for separating 46 biogenic compounds, which, as neurotoxins or as their precursors or derivatives, may be relevant in the pathomechanism of Parkinson's disease. In some cases, the physico–chemical properties of these substances are very similar, in other cases they differ greatly. In order to facilitate their detection in one chromatographic run, ion-pair chromatography was uniquely combined with a gradient elution. A diode array or a dual wavelength detector was used in combination with a fluorescence detector to verify the identity of the compounds.

Keywords: 1,2,3,4-Tetrahydroisoquinolines; Catecholamines; Neurotoxic compounds

1. Introduction

Various HPLC methods may be used to detect components of blood plasma samples which may be relevant to Parkinson's disease (PD), e.g., catecholamines, related biogenic amines, amino acids, β-carbolines, phenyl acetic acids, isoquinolines, etc. [1–9]. The aim of this study was to develop a separation method applicable, as far as possible, to all of the previously mentioned compounds and their related compounds and which would also allow an estimation of their relative concentrations to be made. Due to the differing physico–chemical properties of the above mentioned substances, special HPLC conditions had to be developed to achieve adequate signal heights and acceptable resolution between the compounds in one single chromatographic run.

The elution of the polar, hydrophilic substances, normally the first compounds to be eluted in RP-HPLC, was retarded by ion-pairing at the amine function, thus permitting their separation and detection. In order not to delay elution of the moderately polar and, towards the end of the run, very lipophilic substances, this ion-pairing was reduced during the chromatographic run. At the same time a gradient was used to increase elution capacity by increasing the proportion of organic solvent in the

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Table 1
Composition of gradient AG1: flow-rate: 0.7 ml/min

Time (min)	0	5	40	42	46	52	60
A (%)	95	95	60	0	0	95	95
B (%)	5	5	40	100	100	5	5

Table 3
Composition of gradient AG3: flow-rate 0.8 ml/min

Time (min)	0	10	30	35	40	45	60
A (%)	90	90	55	0	0	90	90
B (%)	10	10	45	100	100	10	10

Table 4
Composition of gradient BG4: flow-rate 0.8 ml/min

Time (min)	0	20	25	30	35	45	47	60
A (%)	100	88	83	70	45	0	100	100
B (%)	0	12	17	30	55	100	0	0

mobile phase. This technique enables the components to be eluted at similar time intervals and has a positive influence on the peak heights. The four gradients finally used (AG1, AG2, AG3 and BG4, see Tables 1–4) differ in their selectivity in various sections of the chromatogram (see Section 3).

However, under certain circumstances it was not always possible to achieve base-line separation of some substances occurring concurrently in some solutions. For this reason and to establish whether, in practise, unresolved related substances were present, UV-spectra were recorded using both a diode array detector and a dual wavelength UV detector, whereby the extinction ratio of the absorptions obtained at a reference wavelength (in this case 240 nm) and at 215 nm was used to provide an additional identification of the substance in question.

Similarly, the use of a fluorescence detector in series increased the sensitivity of detection for many of the substances. The evaluation of the fluorescence intensity in relation to the UV-extinction is an additional tool in establishing the identity of the peaks (see Section 3).

2. Experimental

2.1. Experimental device

2.1.1. System 1

Gradient pump: M 480/Gynkotek HPLC; injection valve; Negretti-Zambra, sample loops 20–50–100–200–500 μl; column: YMC ODS A 5 μm, 150 mm×4.6 mm and YMC PRO C_{18} 5 μm, 150 mm×4.6 mm; detector: UV diode array version 5.30+Opt. DA 320 AB/Gynkothek HPLC, fluorescence spectrometer RF 530/Shimadzu.

2.1.2. System 2

Gradient pump: CM 4000/Milton-Roy; injection valve; Negretti-Zambra, sample loops 20–50–100–200–500 μl; column: YMC ODS A 5 μm, 150 mm×4.6 mm and YMC PRO C_{18} 5 μm, 150 mm×4.6 mm; detector: UVD-160/Gynkotek HPLC and UV detector SM 4000/Milton-Roy, fluorescence spectrometer RF 530/Shimadzu.

2.2. Reagents

Hexanesulphonic acid sodium salt (Fluka No. 52862), ammonium dihydrogenphosphate p.a. (Merck No. 1126), phosphoric acid p.a. (Merck No. 573), ethylenediaminetetraacetic acid sodium salt (Merck No. 8418), sodium sulphite p.a. (Merck No. 6657), formaldehyde solution 37% (Merck No. 1.4003), methanol for gradient-HPLC (Merck No. 6007), water for gradient-HPLC; System 1: initial purification by filtering over silver charcoal, oxidised with alkaline permanganate, decanted, buffered to

Table 2
Composition of gradient AG2: flow-rate 0.8 ml/min

Time (min)	0	10	20	26	34	40	42	47	60
A (%)	93	93	79	70	50	0	0	93	93
B (%)	7	7	21	30	50	100	100	7	7

pH 3 (phosphate) and distilled using glass apparatus; System 2: water prepared by Millipore filtration.

2.3. Reference compounds

The reference compounds used in the analytical procedure and their identification number are listed in Table 5. The identification numbers are also used in column 1 of Tables 6 and 7 and in the attached chromatograms.

2.4. Eluents

The following solvents were used to form the gradient eluents described in Section 2.5: Eluent ''A'': 3 m*M* hexanesulphonic acid (HSA) sodium salt, 12 m*M* $NH_4H_2PO_4$, 3 m*M* H_3PO_4, 50 μmol/l Na_2SO_3, 50 μmol/l EDTA disodium salt, 12 m*M* HCHO. Eluent ''B'': 3 m*M* HSA sodium salt, 12 m*M* $NH_4H_2PO_4$, 3 m*M* H_3PO_4, 50 μmol/l Na_2SO_3, 50 μmol/l EDTA disodium salt–MeOH (91:9, w/w).

2.5. Gradients

A large number of different gradients were tested for their suitability in separating the compounds described in Table 5. The linear gradients (see Tables 1–4) AG1, AG2, AG3 and BG4 were found to be the most suitable.

2.6. Standard test solutions

Test solutions of all substances quoted in Tables 1–4 were prepared in eluent A at concentrations near the UV detection limits. The chromatographic retention behaviour of these substances in the above named eluents with varying concentrations of methanol was tested and the UV-spectra recorded and stored. At the same time, using a serially installed fluorescence detector, the fluorescence was measured at the excitation wavelength of 280 nm and the emission wavelength of 330 nm. Model mixtures with increasing numbers of components were analysed and mean values and retention times in the above mentioned gradients were determined.

Table 5
Reference compounds used for test mixtures and their identification numbers

No.	Compound	No.	Compound
1	Phenylalanine	23	Adrenaline
2	*p*-Tyrosine	31	5,6-Dihdroxytryptamine
3	*o*-Tyrosine	32	5,7-Dihydroxytryptamine
4	*m*-Tyrosine	33	Ethyl-9H-pyrido(3,4,5)indole-3-carboxylate
5	DOPA	34	3-Nitrotyrosine
6	*p*-Tyramine	35	1,2,3,4-Tetrahydroisoquinoline
7	β-Hydroxyphenylethylamine	36	Normetanephrine
8	Octopamine	37	3-Methoxy-4-hydroxyphenylacetic acid
9	3-Methoxytyramine	38	3,4-Dihydroxyphenylacetic acid
10	3-Methoxyphenethylamine	39	Norharman
11	3-Methoxytyrosine	40	Metanephrine
12	β-Phenylethylamine	41	3-Methylisoquinoline
13	5-Hydroxydopamine	42	Isoquinoline
14	6-Hydroxydopamine	43	Harman
15	3-Hydroxy-4-methoxy-phenylethylamine	44	Salsoline
16	Tryptophan	45	Melatonin
17	Tryptamine	46	5-Methoxytryptophan
18	5-Hydroxytryptophane	50	*m*-Tyramine
19c	Creatinine	51	Norsalsolinol
19s	Serotonin	52	*N*-Methylnorsalsolinol
20	6-HydroxyDOPA	53	Salsolinol
21	Dopamine	54	*N*-Methylsalsolinol
22	Noradrenaline	55	6-Hydroxy-1-methyltetrahydroisoquinoline

Table 6
Retention time and response ratio of 22 compounds[a]

No.	AG1	AG2	AG3	Fluor.	BG4	Fluor.	A_{215}/A_{240}	A_{max}
19c	8.1	5.5	5.0	–	4.86	–	5.86	215
22	10.6	7.8	5.8	2.20	5.68	1.90	4.42	200/221
20	11.0	6.1	6.1	0.40	5.24	0.33	6.71	200/293
5	12.3	10.0	7.6	1.56	6.26	1.46	4.61	200/280
23	13.0	8.1	8.1	2.19	7.09	1.99	3.50	221
8	13.6	10.8	8.6	0.48	7.62	0.41	23.00	223
13	15.1	11.9	9.6	0.01	8.36	–	6.57	204
14	16.2	12.9	10.6	0.34	9.94	0.38	9.06	200/291
2	15.6	13.3	11.5	0.68	9.30	0.53	16.50	225
51[b]	15.9	n.t.	n.t.	3.34	9.80	3.15	3.15	n.t.
52[b]	16.1	n.t.	n.t.	3.12	9.91	3.01	n.t.	n.t.
36	16.4	13.4	11.8	1.81	10.50	2.60	2.40	202/228
4	16.8	13.9	12.6	0.62	11.31	0.70	65.00	215
40	18.3	15.4	14.3	2.44	13.55	2.65	2.25	202/228
38	18.5	15.4	14.6	–	14.86	0.06	4.00	204/280
21	17.8	15.7	14.9	1.87	13.61	1.49	6.77	202/280
11	18.0	15.5	15.2	2.38	14.72	2.18	2.53	205/228
53[b]	18.5	n.t.	n.t.	n.t.	14.20	3.32	3.70	n.t.
54[b]	18.5[b]	n.t.	n.t.	n.t.	14.29	3.41	n.t.	n.t.
18	20.4	15.2	17.9	3.57	16.42	3.63	6.95	204
31	21.4	17.1	18.2	0.44	18.06	0.38	7.34	217
1	20.4	18.0	18.6	–	17.90	0.02	84.50	208

[a] Column 1: number of reference compound; columns 2–4 and 6: retention time (diode array detector); columns 5 and 7: ratio (λ_{excit}=280 nm/λ_{emiss}=330 nm)/(A_{215}); column 8: ratio A_{215}/A_{240}; column 9: A_{max} at one or two wavelengths (n.t.=not tested).
[b] Retention time on Milton Roy system.

3. Results

Using model mixtures of the standard solutions of varying concentrations and with differing numbers of components present, the R_T-values of the individual substances were determined. The results obtained from several runs were averaged and documented in Tables 6 and 7. The numbers in column 1 correspond to the component-numbering in Table 5. In columns 2, 3, 4 and 6, the R_T-values in the gradient systems indicated in the header column are given. Column 8 shows the ratio of the extinction-area signal ($mAU\times$ min) at 215–240 nm UV-wavelength. These are used to identify the substances in critical cases (see Section 1) and also to detect artefacts (see Section 4). The values in columns 5 and 7 serve the same purpose and contain the relative fluorescence intensity ($mV\times$min) at a fixed sensitivity (H32) in relation to the $mAU\times$min values of the UV extinction at 215 nm. The slight variations in the figures given in columns 5 (obtained with gradients AG1, AG2 and AG3) and 7 (obtained with gradient BG4) can be explained by the inferior linearity of concentration to recorded value in fluorimetry. For special cases (e.g., when sensitivity has to be increased), the extinction maxima (+preceding bands) of the substances investigated are given.

As mentioned in Section 1, the four gradients demonstrate differing regions of peak densities. The most uniform distribution of all substances is achieved by BG4 (see Fig. 1), with AG3 (see Fig. 4) showing similar characteristics. AG1 produces the lowest peak density in the first and last quartile to the cost of the middle part of the chromatogram. AG2 (see Figs. 2 and 3) lies somewhere between these options. In practise, if one begins with BG4, then it would be advisable to change to, e.g., AG2 or AG1, if a higher resolution is required in the initial range up to ca. 11 min or in the latter range after ca. 30 min.

The quantification of the substance peaks (or for example of their degradation kinetics) was carried out at will by storing the data measured electronically and evaluating against standards at a later date.

Table 7
Retention time and response ratio of 25 compounds[a]

No.	AG1	AG2	AG3	Fluor.	BG4	Fluor.	A_{215}/A_{240}	A_{max}
1	20.4	18.0	18.6	–	17.90	0.02	84.50	208
3	20.0	18.3	18.8	0.72	17.76	0.78	77.00	200/274
34	21.4	19.0	19.0	–	18.89	–	5.10	217
6	21.3	19.2	19.5	0.79	19.34	0.83	33.20	223
32	21.1	20.0	20.7	0.11	20.00	0.15	7.60	219
9	24.6	22.6	24.1	2.65	23.30	2.52	3.40	225
19s	23.6	22.3	24.4	5.2	24.10	4.60	6.77	221
42	23.9	23.9	24.8	–	24.61	–	8.35	228
55[b]	25.2	n.t.	n.t.	2.9	16.37	n.t.	n.t.	n.t.
50[b]	25.6	n.t.	n.t.	0.7	16.30	n.t.	n.t.	n.t.
44	25.6	24.2	25.6	3.72	25.16	3.65	3.64	202/284
7	26.3	26.0	26.7	–	26.50	–	68.00	206
37	26.4	26.5	27.1	0.08	26.30	1.54	2.54	202/280
15	25.7	26.2	27.3	2.6	26.60	2.92	4.00	203/225
46	n.t.	n.t.	30.5	7.3	30.20	3.50	6.00	221/273
41	28.7	29	31.0	0.02	31.00	–	4.14	227
35	23.4	29.3	31.2	–	30.94	–	79.00	228
16	28.3	28.6	33.1	4.02	32.30	3.76	12.23	219
12	32.7	32.1	33.5	–	33.24	–	63.00	206
10	35.3	33.9	35.7	0.62	35.25	0.65	51.00	217/270
17	36.0	34.5	35.9	1.6	35.50	1.65	17.30	217
45	n.t.	n.t.	36.8	4.76	36.23	4.25	5.72	223/278
39	39.2	36.4	37.5	0.01	36.90	0.002	0.69	249
43	41.3	37.0	38.1	n.t.	37.43	0.01	0.56	248
33	54.8	42.2	42.6	n.t.	42.04	0.02	0.85	217

[a] Column 1: number of reference compound; column 2–4 and 6: retention time (diode array detector); column 5 and 7: ratio (λ_{excit}=280 nm/λ_{emiss}=330 nm)/(A_{215}); column 8: ratio A_{215}/A_{240}; column 9: A_{max} at one or two wavelengths (n.t.=not tested).
[b] Retention time on Milton Roy system.

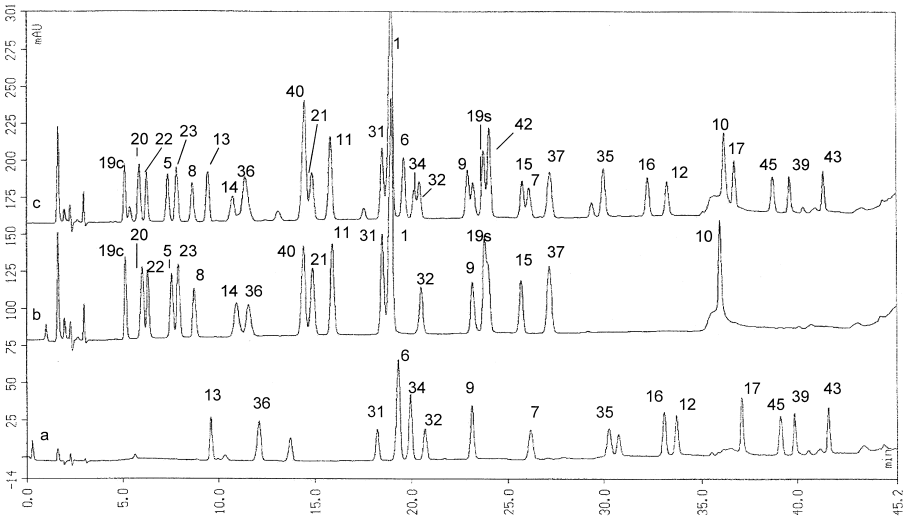

Fig. 1. Chromatogram of three test mixtures: mixture c=a+b (λ_{emiss}=215 nm), gradient system BG4.

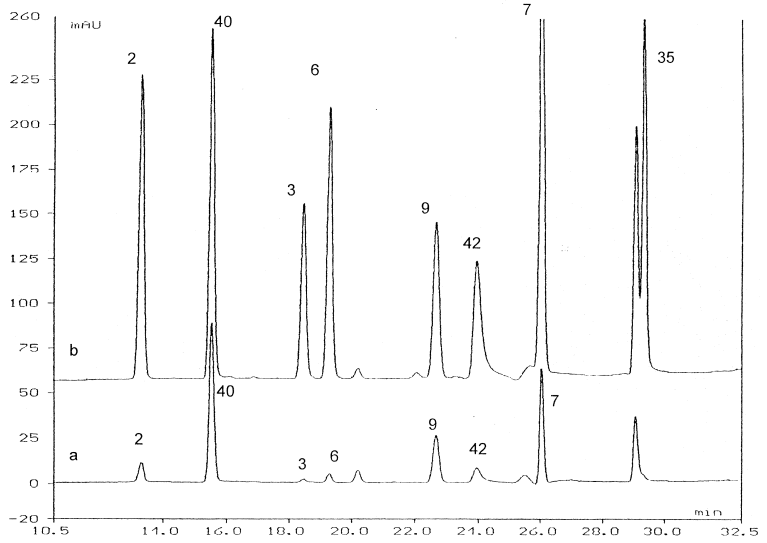

Fig. 2. Chromatogram of a test mixture (a: λ=240 nm, b: λ=215 nm), gradient system AG2.

Complete chromatographic and optical data of the substances 50–55 most recently received are not yet available. In Table 8, they will be complemented by comparative measurements of the limit of detection between electrochemical detection (ED), fluorescence detection (FD) and UV-extinction at 240 nm.

4. Discussion

Robustness of the method was demonstrated in model solutions containing up to 30–35 of the tabulated substances. They were identified in system 1 (DAD detection) by their UV-spectra with match

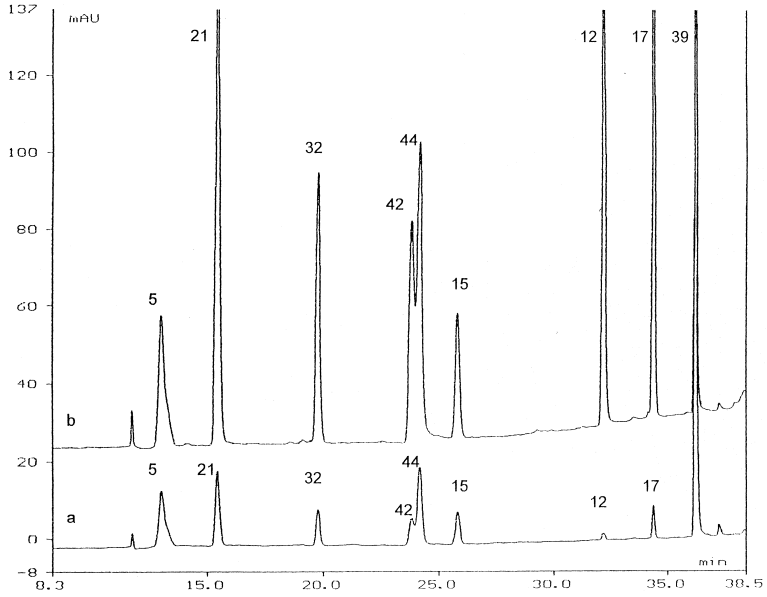

Fig. 3. Chromatogram of a test mixture (a: λ=240 nm, b: λ=215 nm), gradient system AG2.

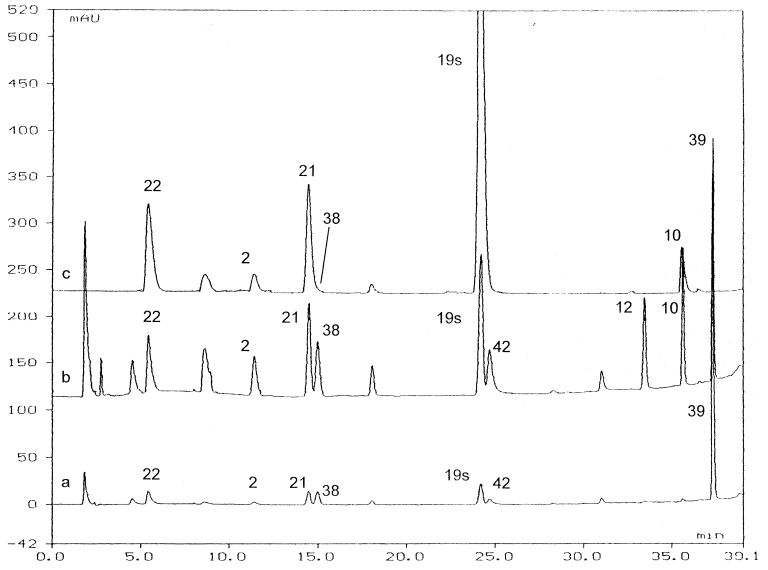

Fig. 4. Chromatogram of a test mixture [a: λ=240 nm, b: λ=215 nm, c: FD (λ_{excit}=280 nm/λ_{emiss}=330 nm)], gradient system AG3.

factors of >996/1000 (as coincidence criteria), by their R_T-values and by the ratio of fluorescence emission to UV-extinction value (215 nm). In system 2, identification was carried out using two-channel detection (extinction ratio) instead of the DAD measurement, otherwise in the same way as in system 1.

The two systems were installed at different geographic locations (system 2 in the University of Ulm and system 1 in another laboratory, situated 30 km south of Ulm, in Erolzheim). Comparative measurements were carried out on both systems by various operators and over a period of 7 months.

Table 8
Limit of detection (LOD) of some compounds by UV and FD detection[a]

Compound	LOD-ED (pmol)	LOD-FD (pmol)	LOD-UV/240 (pmol)
DOPA		1.5	6
Salsolinol	0.05–0.08 [3] 0.01 [2]	1	5
6-Hydroxy-1-methyl-1,2,3,4-THIQ		1	5
Salsolin[b]		1	5

[a] Signal-to-noise ratio greater than 3.
[b] 6-Hydroxy-7-methoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline.

Many of the substances under investigation are unstable in solution and especially in the presence of other substances. The artefacts formed were detected as they were determined to be unknown substances, as they could not be identified by evaluation of their spectra (DAD), extinction ratios or fluorescence ratios.

Variation in the retention times only exceeded 1%, if reconditioning was unsatisfactory. R_T variations also occurred if the unit had not been used for some time (e.g., overnight), if a blank run had not been carried out, if the ambient temperature exceeded the normal range of 20±2°C and especially when a spent column was changed.

The reproducible peak symmetry of the test substances may be attributed to a large degree to the suitability and efficiency of the YMC columns used. However, uneven temperature effects on the column, such as direct sunlight, draughts etc. can have a detrimental effect on the results.

The above recommendation to carry out a blank run combines the possibility of being able to check R_T-values and to determine whether any ''ghost peaks'' are present. These usually originate from impurities in the reagents or solvents (such as water and methanol) and can lead to false positive results in identifying the presence of analytical components.

It is not clear, whether the small amount of formaldehyde in the eluent A reacts with some of the test substances (see Ref. [9]).

With respect to the limit of detection (LOD), it was shown experimentally, that using a sample injection of 50–100 μl (as opposed to the usual 20-μl sample injection) did not result in a loss in resolution, even for the early eluting substances; this being attributable to the gradient elution. For late-eluting components it is advisable to increase the sample injection size to 100–300 μl, thus achieving a corresponding increase in the level of detection without peak widening interfering with the chromatogram.

The currently preferred oxidative electrochemical detector achieves a much lower LOD for the phenolic hydroxy groups. This advantage over the UV and fluorescence detection is lost by not being able to use the gradient elution technique. Above all, it does not achieve the aim of being suitable for general screening purposes.

The measuring techniques described here and tried out on model solutions have been extended to experimental measurements of the components of human plasma. Examples of these results are presented in a preceeding paper [10].

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