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Reversed-phase ion-pair chromatography coupled to electrospray ionisation mass spectrometry by on-line removal of the counter-ions

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Abstract

A strong anion-exchanger was used as a trapping column to perform on-line coupling of ion-pair chromatography with electrospray ionisation mass spectrometry. Ion-pairing reagents were used to retain polar analytes of low molecular mass away from the solvent front in reversed-phase LC. The trapping column enabled removal of the non-volatile counter-ions from the mobile phase prior to detection, so that the electrospray process could be performed with favourable ionisation conditions and without contamination of the interface. The efficiency of the trapping process was studied for 1-octanesulfonic acid and sodium dodecyl sulfate as ion-pairing reagents. Using this on-line trapping method, biopterin and guanidine could be retained with a $k'>2$ and detected by electrospray mass spectrometry with a stable signal.

Keywords: Ion-pair chromatography; Liquid chromatography–mass spectrometry; Detection, LC; Biopterin; Guanidine

1. Introduction

Liquid chromatography coupled to mass spectrometry (LC–MS) has become an important tool in biomedical analysis. A number of different ionisation techniques can be used in order to transfer the analytes from liquid phase to gas phase, to enable their introduction to the mass spectrometer. One of the most popular interfaces is electrospray ionisation (ESI), which provides soft ionisation and has the ability to ionise a wide variety of different compounds. The electrospray process is compatible with many buffers commonly used in liquid chromatography, however, with the limitation that the solvents and additives have to be volatile to maintain a stable spray performance [1]. In addition, the buffer additives should typically be below 20 m*M* [2] in concentration, since higher electrolyte concentrations provoke signal instability and ultimately electrical breakage [3]. It is also known that non-volatile substances, like most ion-pairing reagents and inorganic buffer additives, destabilise the electrospray process [4] and contaminate the interface [3]. This is especially limiting in ion-exchange chromatography (IEC) and ion-pair chromatography (IPC).

IEC and IPC are based on charged analyte properties, and are frequently used for analysis of biologically interesting substances, which are often polar and of low molecular mass. Biological distribution and metabolism of a substance can be

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determined by use of a compound analogue labelled with radionuclides such as ^{3}H, ^{11}C and ^{14}C or stable isotopes like ^{2}H and ^{13}C. In order to monitor yields during synthetic method development, to determine purity or to perform metabolic studies, it is important to use proper analytical separation methods and selective detection. Radiodetection and mass spectrometry are two of the most selective detectors for such analyses and it has therefore been of interest to couple IEC and IPC techniques to electrospray ionisation mass spectrometry.

In ion-exchange chromatography, where the separation is based on a charged analyte's affinity for charged sites on the column, high buffer concentrations are commonly required for elution of the analytes. Due to the resulting high electrolyte concentrations, ion-exchange chromatography is normally not compatible with electrospray ionization. Suppressor membranes have therefore been used post-column to reduce the electrolyte content prior to detection [2,5–8]. This device has proved efficient in removing large amounts of interfering salt ions, as long as care is taken not to induce a high back pressure (i.e. not above 2 MPa) [6,7].

The application of reversed-phase ion-pair chromatography (R-IPC), as compared to ion-exchange chromatography, to the separation of charged analytes has the advantage that both neutral and ionic species can be separated. The electrospray ionisation process, however, is not compatible with large amounts of ion-pairing reagents [2,9,10], as analyte suppression occurs in the detection when the buffer contains non-volatile compounds [4,11]. Several methods have been presented to overcome this limitation. For example, the ionic detergent sodium dodecyl sulfate (SDS) has been removed successfully on-line from tryptic digest samples prior to analysis [12–15]. Others have replaced non-volatile additives in the mobile phase by volatile compounds [16–18], such as heptafluorobutanoic acid, tridecafluoroheptanoic acid and nonadecafluorodecanoic acid. Such mobile phases are directly compatible with ESI–MS, though the use of ion-pairing reagents in the mobile phase will cause very high chemical background [18] and there is currently a limited range of volatile counter-ions.

The use of non-volatile ion-pairing reagents in the mobile phase requires some post-column removal system. Different valve-switching techniques have been used to remove the counter-ions after separation and prior to detection [19–21]. Such phase-system switching can enable high selectivity and remove large amounts of ion-pairing reagents [21] or salts [20], but the system is discontinuous and can generate band broadening since it involves adsorption of analytes followed by desorption under back-flush. In addition to the methods described above, new electrospray interface designs have been introduced which are more tolerant of non-volatile components in the spray. By use of an orthogonal spray [22], the interface becomes less contaminated by the non-volatile substances.

In this study, reversed-phase ion-pair chromatography was coupled to ESI–MS by an on-line trapping method for the separation and analysis of polar analytes. In order to remove the non-volatile components from the buffer prior to the mass spectrometer inlet, a strong anion-exchanger trapping column was coupled between the separation column and the electrospray probe. The efficiency of the trapping column for the removal of non-volatile ionic species was investigated as well as the stability of the resulting signal and preservation of chromatographic resolution. The trapping process was evaluated using negatively charged counter-ions of two different chain lengths, while separation was performed with the ion-pairing reagent that gave adequate retention time for the analyte in question. The trapping method was developed with biopterin as a model analyte. The technique was then used to analyse synthetic products from radiolabelling syntheses, by coupling of a radiodetector to the system.

2. Experimental

2.1. Materials

Formic acid of analytical grade was from E. Merck (Darmstadt, Germany). Methanol of ultra gradient grade from Fisons (Loughborough, UK) and nanopure water (Elga Maxima, Bucks, UK) were used for mobile phases. 2-Amino-6-(1,2-dihydroxypropyl)-3*H*-pteridin-4-one (L-biopterin) was purchased from Schircks Laboratories (Jona, Switzerland). Fused silica tubing was obtained from Poly-

micro Technologies (Phoenix, AZ, USA). Ammonium formate was purchased from Aldrich (Stockholm, Sweden). The ion-pairing reagents were 1-heptanesulfonic acid sodium salt monohydrate (SHS) from Sigma–Aldrich (Stockholm, Sweden), 1-octanesulfonic acid sodium salt (SOS) from Sigma and SDS from Fluka Chemie (Tokyo, Japan). The [^{11}C]-guanidine synthesis was performed according to procedures described elsewhere [23].

2.2. Packed capillary liquid chromatography

In the biopterin analyses, three different buffers were used; (A) 3 m*M* SHS and 5 m*M* formic acid in water–methanol (95:5, v/v), (B) 1 m*M* SOS acid and 5 m*M* formic acid in water–methanol (95:5, v/v) and (C) 3 m*M* SDS and 5 m*M* formic acid in water–methanol (70:30, v/v). All guanidine analyses were performed in buffer (A). The mobile phases used in the trapping experiments all consisted of 5 m*M* formic acid together with the ion-pairing reagent and methanol content according to Table 1.

All the columns were packed in 500 μm I.D. polyether ether ketone (PEEK) tubing. A reversed-phase separation column of 50 mm×500 μm I.D. was packed with Kromasil 5 μm C_{18} material (Phenomenex, CA, USA) using supercritical carbon dioxide as the packing material carrier according to previously published procedures. [24] The anion-exchanger consisted of MiniQ material, non-porous 3 μm monodispersed hydrophilic polyether particles, which was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The material was packed in PEEK tubing of 40 mm×500 μm, in a methanol slurry from a reservoir in an ultrasonic bath. The packing was performed using LC pumps while care was taken not to exceed 10 MPa, which was the maximum operating pressure given by the manufacturer for the packing material. The column end frits consisted of stainless steel filters with 2 μm porosity (Skandinaviska Genetec, Kungsbacka, Sweden). The analytical column and the anion-exchanger were coupled with 20 mm PEEK tubing (0.13 mm I.D.).

A Beckman 126 solvent delivery module (Beckman Instruments, CA, USA) was used for all analyses and were run at a flow-rate of 10 μl min^{-1}. Biopterin samples were prepared by dissolving the substance in 0.15 *M* formic acid. Before injection the samples were diluted with mobile phase. A volume of 2 μl sample was injected with CMA autosampler (CMA/Microdialys, Stockholm, Sweden). A low dead volume transfer line was made from 30 cm fused-silica tubing (40 μm I.D.×110 μm O.D.) which connected the anion-exchange column with the electrospray probe.

2.3. Radiodetection

Radiodetection was performed with a Flow-Count β^+-detector (Bioscan, Washington, DC, USA). The fused-silica capillary, that connected the trapping column with the electrospray probe, was passed through the radiodetector prior to the mass spectrometer as shown in Fig. 1.

Table 1
Ion-pair breakthrough on an anion-exchange column[a]

Ion pair reagent	Conc. (m*M*)	Organic mod. (%)[b]	m/z[c]	Breakthrough time (min)	Loaded mass (μg)
SOS[d]	1	5	193	161±12[f]	348±26[f]
SOS[d]	3	5	193	55±1[g]	356±7[g]
SOS[d]	1	15	193	154±4[g]	333±9[g]
SDS[e]	1	5	265	142±6[g]	409±17[g]

[a] A flow-rate of 10 μl min^{-1} was used and all mobile phases contained 5 m*M* formic acid.
[b] Methanol was used as organic modifier in all experiments (v/v).
[c] Electrospray ionisation in negative mode.
[d] 1-Octanesulfonic acid, sodium salt.
[e] Dodecyl sulfate, sodium salt.
[f] The capacity and reproducibility of counter-ion removal were determined by four separate loadings on the same column.
[g] The capacity and reproducibility of counter-ion removal were determined by two separate loadings on the same column.

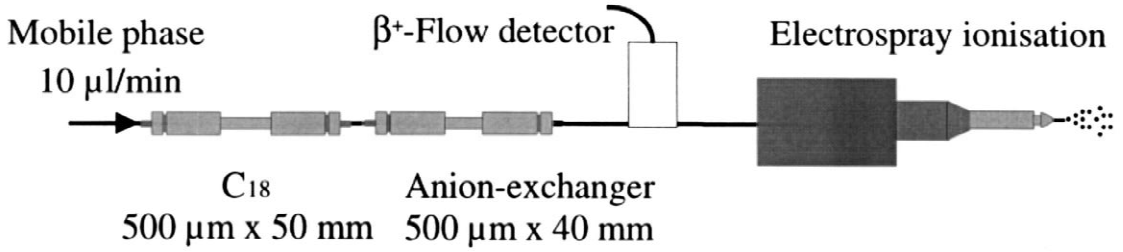

Fig. 1. Schematic diagram of the on-line coupling of an anion-exchange trapping column in-between analytical capillary LC and electrospray ionisation mass spectrometry.

2.4. Mass spectrometry

The mass spectrometer was a VG Quattro (Micromass, Manchester, UK). Pneumatically assisted electrospray ionisation was used in all experiments, with the probe modified by an empty fused-silica tubing (connected to the anion-exchange column) inserted inside the original electrospray needle. The tubing outlet was positioned even with the steel capillary outlet allowing electrical contact on the very tip of the steel capillary upon elution. Detection parameters for biopterin in the mass spectrometer were determined by flow injection analysis of a biopterin solution of biopterin containing 5 m*M* formic acid at a flow-rate of 20 µl min^{-1}. The protonated molecule of biopterin could be detected at *m/z* 238 in positive electrospray ionisation. The capillary voltage was set to 2.5 kV and the cone voltage to 30 V.

Tandem mass spectrometry was used for all biopterin analyses using the precursor-ion/product-ion pair *m/z* 238–*m/z* 220. Mass spectra were also acquired for the three ion-pairing reagents. The deprotonated molecules $[M-H]^-$ of the substances were found in ESI^- at *m/z* 179 (SHS), *m/z* 193 (SOS) and *m/z* 265 (SDS) and the sodium adducts $[M+Na]^+$ in ESI^+ at *m/z* 225 (SHS), *m/z* 239 (SOS) and *m/z* 311 (SDS). Guanidine was determined at *m/z* 60.

3. Results and discussion

3.1. Chromatography

Reversed-phase ion-pair chromatography (R-IPC) can be used to separate both neutral and ionic analytes in the same analysis. The ion-pairing reagent is used to retain polar ionic analytes that otherwise would be eluted in the solvent front in reversed-phase chromatography. In this study two low molecular mass polar analytes, biopterin and guanidine, were analysed by R-IPC. Buffers at low pH were used in order to keep the analytes charged to promote the ion-pairing process. The mobile phases were therefore prepared with the ion-pairing reagent of interest, together with 5 m*M* of formic acid and the column was allowed to equilibrate overnight. It was found that biopterin was well separated from the solvent front on a reversed-phase C_{18} column using mobile phases containing any of the three following compositions; (A) 3 m*M* of SHS and 5 m*M* formic acid in water–methanol (95:5, v/v), (B) 1 m*M* of SOS and 5 m*M* formic acid in water–methanol (95:5, v/v) or (C) 3 m*M* SDS and 5 m*M* formic acid in water–methanol (70:30, v/v). The analyte guanidine was analysed with a mobile phase (A) using SHS as the counter-ion.

3.2. Effect of non-volatile components in the spray

Prior to the coupling of a trapping column to the system, the extent of instability that ion-pairs of two different chain-lengths (SDS and SHS) were causing in the ionisation process was evaluated. This was performed by infusion of a mobile phase consisting of 3 m*M* of SDS at a flow-rate of 20 µl min^{-1}. The signal from the ion-pairing reagent was determined every 30 min by detection at *m/z* 311 in the mass spectrum, which corresponds to the molecular ion of SDS from sodium attachment $[M+Na]^+$ [25]. As

expected, the signal intensity rapidly decreased when the mobile phase contained the ion-pairing reagent as could be seen by monitoring the mass spectrum of SDS (Fig. 2a). The measured signal was seen to disappear at times only to recover somewhat again. This observation was believed to be a result of partial blocking of the high voltage lens, which omitted all ions from detection. The drying gas flow in the ion source will counteract the blocking process, which could explain why the signal occasionally was recovered. After seven hours though, the signal had disappeared altogether and a white layer of powder was visible on the sampling cone as well as on the high voltage lens. When a smaller ion-pairing reagent (SHS) was used at a reduced concentration (1 m*M*) the signal was less unstable, but the signal intensity was constantly decreasing (Fig. 2b) and the cone was again visibly contaminated. It was clear from these experiments that a reliable analysis was not possible while these ion-pairing reagents were present in the detection process.

3.3. Trapping process

The capacity and reproducibility of an anion-exchanger to trap the counter-ion prior to detection was evaluated for two different ion-pairing reagents, by use of packed capillary columns (40 mm×500 μm I.D.). The anion-exchange columns were of strong quaternary ammonium-type, made from nonporous monodispersed MiniQ beads, with a particle size of 3 μm. The capacity for trapping of the ion-pairing reagent was determined by passing the mobile phase, containing the ion-pairing reagent and 5 m*M* of formic acid, through the column at a flow-rate of 10 $\mu l\,min^{-1}$. The trapping process was monitored with the mass spectrometer, by determination of the ion-pairing reagent of choice in the negative electrospray ionisation mode. The time of breakthrough was

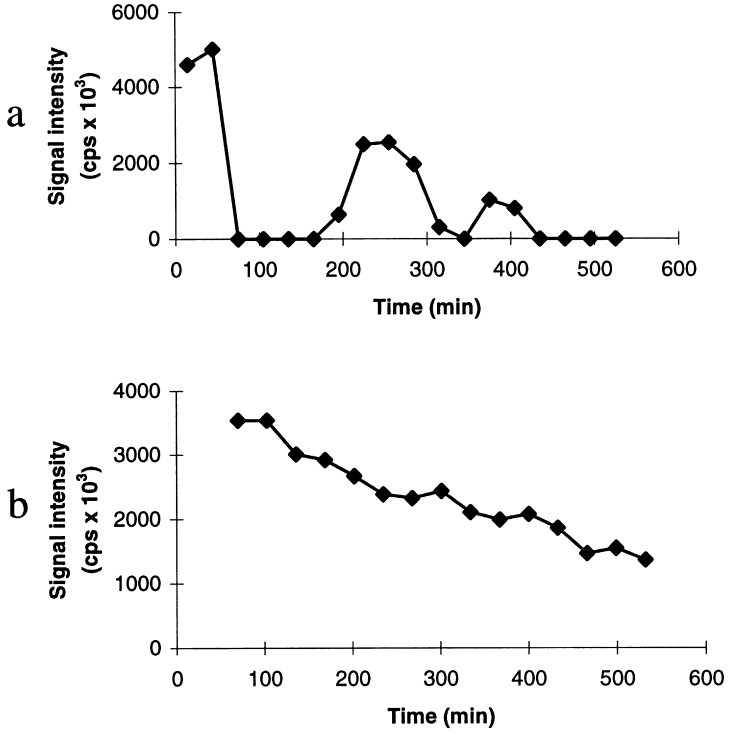

Fig. 2. Signal intensity as a function of time for continuous infusion at 10 $\mu l\,min^{-1}$ of mobile phases containing ion-pairing reagents. Conditions: (a) 3 m*M* SDS and 5 m*M* formic acid in water:methanol (95:5, v/v) and (b) 1 m*M* SHS and 5 m*M* formic acid in water:methanol (95:5, v/v).

easily determined, as the signal became very intense once the ion-pairing reagent was entering the mass spectrometer. The capacity was determined for mobile phases containing 1 m*M* SOS, 3 m*M* SOS and 1 m*M* SDS as shown in Table 1. Breakthrough of 1 m*M* SOS appeared at 160 min (corresponding to 350 μg or 1.6 ml of 1 m*M* SOS solution) and when the concentration of SOS was increased from 1 to 3 m*M*, the breakthrough was three times faster, as expected (Table 1). The trapping efficiency with a higher methanol content in the mobile phase was also evaluated. It was found that the time for breakthrough did not change significantly (i.e. 154 min, 330 μg and 1.5 ml), when the methanol content was increased from 5% to 15% (v/v).

3.4. Cleaning process

After breakthrough of the ion-pairing reagent (SOS), the anion-exchangers were washed at a flow-rate of 10 μl min^{-1} with 50 m*M* ammonium formate solutions for one hour followed by 10 min of water. This procedure completely regenerated the trapping columns. During analysis it was important to keep the pH of the eluate constant, to maintain a consistent electrospray response. Formic acid was therefore monitored at m/z 45 during the run. Since the anion-exchange column had been previously washed with formate ions, the ion intensity of this particular ion remained constant during the whole run as did the pH.

A sodium hydroxide solution could also be used for complete regeneration of the trapping columns. One disadvantage, however, was that the concentration had to be low as the packing material had a pH stability of 3–11 according to the manufacturer. Several hours were required for adequate washing, when a 6 m*M* sodium hydroxide solution was used. Another disadvantage with this procedure was that it would take a long time before the formate ion would break through and during this time the ESI response was found to be lower than during the rest of the run.

3.5. LC–MS coupling

In order to couple the separation and the trapping columns together, the analytical column was first equilibrated with mobile phase and the trapping column thoroughly washed with ammonium formate. The anion-exchange column was then placed between the separation column and the electrospray probe as shown in Fig. 1. In order to reduce chemical background noise, the biopterin analyses were all performed by use of tandem mass spectrometry. Biopterin was injected every 20 min and detection was performed by multiple reaction monitoring (MRM) of the precursor-ion/product-ion pair m/z 238–m/z 220. Between biopterin injections the formate ion was monitored at m/z 45 to verify that it was stable during the entire run. In addition, the counter-ion was monitored every 20 min at m/z 179 (SHS), m/z 194 (SOS) or m/z 311 (SDS) to determine the time of ion-pairing reagent breakthrough.

The peak areas were determined for repetitive injections of biopterin and the relative standard deviation (RSD) was calculated to be 3% ($n=5$). Retention times were reproducible and calculated to have an RSD of$<1\%$ when biopterin was retained with $k'=4$. Chromatograms for biopterin were compared with and without the trapping column in the system. It could be seen that the dead time of the system was increased by 30 s with the trapping column inserted and the efficiency, determined at half peak height, was reduced from 32 000 plates m^{-1} to 29 000 plates m^{-1} upon addition of the extra column.

3.6. Analysis of radiolabelled compounds

As an example, the ion-pairing reagent trapping technique was used to analyse products from synthesis development of compounds labelled with short-lived β^+-emitting radionuclides (e.g. ^{11}C, $t_{1/2}=$ 20 min) for use in positron emission tomography (PET) [26]. Analysis of such synthetic products are frequently performed with liquid separation techniques by use of a radiodetector in series with an ultraviolet (UV) detector, to correlate the labelled product to a reference compound. In analyses of the raw product from synthesis of ^{11}C-labelled guanidine [23] separation of the reaction components could be performed using reversed-phase ion-pair chromatography, but the guanidine compound itself was difficult to detect with ultraviolet (UV) detection. It was

therefore interesting to couple this technique to electrospray mass spectrometry detection. A radiodetector was thus coupled to the system between the trapping column and the mass spectrometer as shown in Fig. 1, in order to analyse the radiolabelled compounds. The fused-silica tubing used for connection to the electrospray probe was then passed through the β^+-flow detector. Since the peak width in the radio-chromatogram depends on the length of tubing that passes the detector cell, corresponding to a volume of about 0.18 μl in this experiment, the radiodetector gave broader peaks than the subsequent mass spectrometer (Fig. 3). The synthetic mixture was then analysed by R-IPC, with the trapping column coupled in series using mobile phase (A) containing SHS as the counter-ion. Guanidine was determined by selected ion monitoring at m/z 60. The mass chromatogram showed one early eluting peak and one at 7.3 min. By addition of unlabelled guanidine it was determined that this compound corresponded to the late eluting peak and that only a small fraction of the total radioactivity corresponded to guanidine in that particular sample (Fig. 3). By use of this analysis method we now have the possibility to analyse the reaction mixtures, with the aim of improving the synthetic yields.

4. Conclusions

The use of a strong anion-exchange trapping column made it possible to couple ion-pair chromatography to electrospray mass spectrometry, by removal of the ion-pairing reagents before detection. Biopterin could be repeatedly analysed for 3 h without interruption or decrease in signal by use of a separation column coupled to the anion-exchanger. The trapping columns could be regenerated between analyses and the addition of this column to the analytical system caused only a small efficiency decrease from 32 000 plates m^{-1} to 29 000 plates m^{-1}. The method was used to determine products from radiolabelling synthesis by use of radiodetection and electrospray mass spectrometry in series.

The trapping process proved to work with two ion-pairing reagents of different chain-lengths and at varying concentrations. In possible future studies, it would be of interest to evaluate other anion-exchange materials as well as cation-exchangers. For applications that would require more than three h of analysis time it should be possible to alternate the use of two trapping columns by use of a column switch. One column could then be used while the other one was being regenerated.

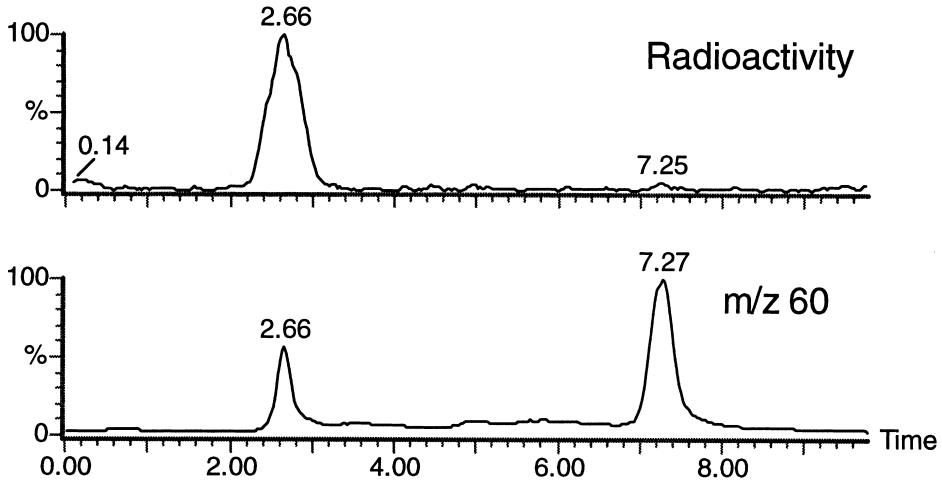

Fig. 3. Reversed phase ion-pair chromatography of a raw product from synthesis of ^{11}C-labelled guanidine. SHS was used as ion-pairing reagent (3 m*M*) to retain guanidine on the separation column and was removed prior to detection by trapping on an anion-exchange column. Detection was performed with a β^+-flow and an ESI–MS detector in series. Guanidine eluted at 7.3 min and could be correlated to a minor small fraction of the radioactivity.

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