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Capillary Electrophoresis with Postcolumn Infectivity Assay for the Analysis of Different Serotypes of Human Rhinovirus (Common Cold Virus)

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Differentiation of virus serotypes with capillary zone electrophoresis was demonstrated. For four serotypes of human rhinovirus (HRV2, HRV14, HRV16, HRV49), different electrophoretic mobility was achieved at pH 8.3 (borate/boric acid buffer, 100 mmol/L). Addition of detergent (Triton X-100-R, deoxycholate, and/or SDS) to the background electrolyte was required for reduction of wall adsorption and improvement of peak shape. A major nonviral contaminant, present in all virus samples, was best separated from the viral peaks with 10 mmol/L SDS as additive. The method allowed detecting serotypes HRV16 and HRV49 in crude, partially purified virus preparations. An infectivity assay carried out off-line with fractions collected at the capillary outlet enabled the sensitive and biospecific identification of the peaks of HRV2 and HRV14.

Capillary zone electrophoresis (CZE) has gained importance for analyzing and quantifying biological macromolecules such as proteins and nucleic acids.[1,2] It was demonstrated that even more complex macromolecular assemblies such as a human rhinovirus (HRV) could be characterized by CZE.[3−6] HRVs are particles with a molecular mass of $\sim 8 \times 10^6$ Da and a diameter of 30 nm. They are composed of four viral capsid proteins (VP1−VP4), with the icosahedral protein shell enclosing an RNA genome.[7] To rapidly assess the purity of preparations of human rhinovirus serotype 2 (HRV2), an analytical CZE method was developed which is now routinely used in our laboratory.[5] Due to the resistance of native HRV2 against low concentrations of SDS,[8] this detergent could be used to prevent capillary wall adsorption and proved most effective in resolving the viral peak from contaminants. The method has the potential for quantification of native virus and allows evaluating the amount of contaminants originating from cell debris and serum components used in tissue culture.

HRVs undergo conformational modifications to subviral particles upon exposure to elevated temperature or to low pH leading to loss of VP4 and subsequently to release of the genomic RNA.[9] Furthermore, even prolonged storage at 4 °C results in viral inactivation; it was therefore desirable to identify in the electropherograms the subviral particles originating from this process. The surface charge of the particles differs from that of native virions giving rise to a different behavior in capillary electrophoresis. As SDS disrupts subviral particles, a ternary detergent mixture was especially developed to prevent aggregation and capillary wall adsorption. Taking this into account, successful separation of subviral particles from intact virus was obtained.[6] In both cases, the analytes were unambiguously identified by their characteristic spectra and, more relevant, by precolumn immunoaffinity reactions with aggregating monoclonal antibodies directed against native virus and subviral particles, respectively.

So far, the suitability of CZE was demonstrated only for one particular HRV serotype, i.e., HRV2. However, more than 100 rhinovirus serotypes are known; on the basis of the available structure and sequence information, it can be assumed that they all have a high degree of similarity on the level of the amino acid sequence of their capsid proteins. Nevertheless, surface loops recognized by neutralizing antibodies show variability and are the basis for the serotypic classification. The serotypes are divided into a major group (91 serotypes) and a minor group (10 serotypes) based on their specific binding to intercellular adhesion molecule 1 (ICAM-1) or to members of the low-density lipoprotein receptor (LDLR) family, respectively, which are used as receptors for cell entry.[10,11] We thus reasoned that specific surface properties might be associated with members of the two receptor

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groups and asked whether the subtle differences suffice for a different migration behavior in the electric field.

Using three other rhinovirus serotypes, HRV14, HRV16, and HRV49 in addition to HRV2, we report here on an investigation of the migrational behavior of these HRVs in CZE. This is the one major goal of the present paper. HRV2 and HRV14 were extensively purified by sucrose density gradient centrifugation, whereas in the case of the other serotypes, infected cell culture supernatants were subjected to a high-speed centrifugation and the resuspended virus pellets were used; thus, the last purification step was omitted.

The second goal is to demonstrate the possibility of a post-column infectivity assay for the confirmation of peak identity. This assay should allow substantial enhancement of the selectivity and sensitivity of detection, as theoretically one infectious viral particle is enough to cause cell lysis. It is based on the collection of individual fractions at the capillary outlet (in a way similar to that described by Ebersole and McCormick[12] for bacteria) and incubation with subconfluent HeLa cells. The presence of virus resulted in cell death.

EXPERIMENTAL SECTION

Apparatus. The CZE measurements were carried out with a ^{3D}Capillary Electrophoresis System (Hewlett-Packard, Waldbronn, Germany), equipped with a built-in diode-array detector. All CZE separations were performed in a fused-silica capillary (Polymicro Technologies, Phoenix, AZ) with 60.0 cm/51.5 cm length and 50 μm i.d. The capillary was packed in a standard HP cassette and thermostated at 20 °C during all experiments.

Prior to injection and between all runs, the capillary was rinsed with 0.1 mol/L NaOH, water, and background electrolyte (BGE) for 2 min each. All solutions were centrifuged for 2 min in a tabletop centrifuge at 5.000*g* prior to CZE analysis. Injection was accomplished by application of 50 mbar pressure to the inlet vial for 9 s. Analyses were conducted in the positive polarity mode at 25 kV.

The detector signals were recorded at 205 and 260 nm. Fast spectral scanning mode was used in specified cases. Data collection and analysis was conducted using Hewlett-Packard Chemstation Software.

Fraction Collection. The CZE system was programmed to change the outlet vials every minute starting at 3.0 min after the first application of voltage. Each outlet vial contained 20 μL of BGE to maintain current during analysis. Identical fractions of two consecutive runs were collected into the same vial.

Reagents. Chemicals were obtained from E. Merck (Darmstadt, Germany) and were used without further purification. BGE was 100 mmol/L boric acid, adjusted to different pH values with 1 mol/L NaOH as specified in the text. Additives were either 10 mmol/L SDS (0.26% w/v), 12.5 mmol/L sodium deoxycholate (0.5% w/v), or a ternary mixture of detergents (0.5% sodium deoxycholate, 0.05% SDS, 0.5% Triton X-100-R; all w/v).

Human rhinovirus serotype 2 and serotype 14 were produced and purified from infected cell pellets as described previously.[13] For HRV16 and HRV49, sucrose gradient centrifugation was omitted and the resuspended virus pellet was used directly.

For HRV2 and HRV14, the concentration was adjusted to between 0.1 and 0.25 mg/mL total protein. All samples were prepared in 1:2 diluted BGE without detergents added and were supplemented with *o*-phthalic acid (20 μg/mL) as an internal standard.

Infectivity Assay. For maintenance of Rhino HeLa cells (Flow Laboratories), minimal essential medium containing 10% fetal calf serum (Life Technologies) supplemented with penicillin and streptomycin was used. Infection medium contained 30 mmol/L $MgCl_2$ and 2% fetal calf serum. Cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. Infections were carried out at 34 °C. Cells grown in a 96-well microtiter plate (in 100 μL of medium/well) to 60% confluency were challenged with 100 μL of serial 3-fold dilutions of the viral samples to be assayed: 150 μL of infection medium were mixed with 2 μL of the respective fraction collected from the outlet of the capillary. A 100-μL aliquot was added to the first well, whereas the remaining 50 μL was further diluted and transferred to the respective wells. The plate was incubated for 5 days at 34 °C. Cells remaining attached to the plastic surface were stained with a 0.1% solution of crystal violet in water after the liquid had been removed. The plate was then washed with water and colorless wells were scored positive for virus infection. Wells appearing stained as a result of lack of infection were scored negative.

RESULTS AND DISCUSSION

Assessment of Buffers for the Differentiation of HRV2 and HRV14. *Modified RIPA Buffer.* Radioimmunoassay buffer (RIPA buffer;[14]) contains a ternary mixture of detergents (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100) that has proved valuable in immunoprecipitation, as it does not inhibit the interaction between various antigens and their cognate antibodies but at the same time minimizes unspecific interactions. This buffer has also been successfully used for *Staphylococcus aureus*-aided immunoprecipitation of HRV2 and of its subviral particles.[15] Further, after modification (see Experimental Section), it was found suitable for the separation of HRV2 from subviral particles in CZE.[6] It was thus investigated whether HRV2 and HRV14 would migrate with different mobilities in this BGE formulation. As already shown in earlier work,[6] HRV2 was well resolved from contaminants and gave rise to a major peak at 4.2 min (Figure 1B). Analysis of purified HRV14 in the same system resulted in a main peak with 3.8 min migration time (Figure 1A). Comparison of the UV spectrum of this peak with that of HRV2 (whose identity was substantially confirmed previously[5]) revealed their virtual identity (Figure 2). Both spectra exhibit a local maximum at 260 nm, indicating the presence of nucleic acid. This strongly suggests that the main peak in the sample of HRV14 indeed corresponds to virus. As also depicted in Figure 2, the spectrum of the main contaminant (C) was clearly different. The identity of the peak tentatively assigned to HRV14 was then confirmed by infectivity assay (see below).

In this particular BGE, peak identification was complicated by the fact that the major contaminant present in the HRV2 preparation exhibits almost the same mobility as the peak ten-

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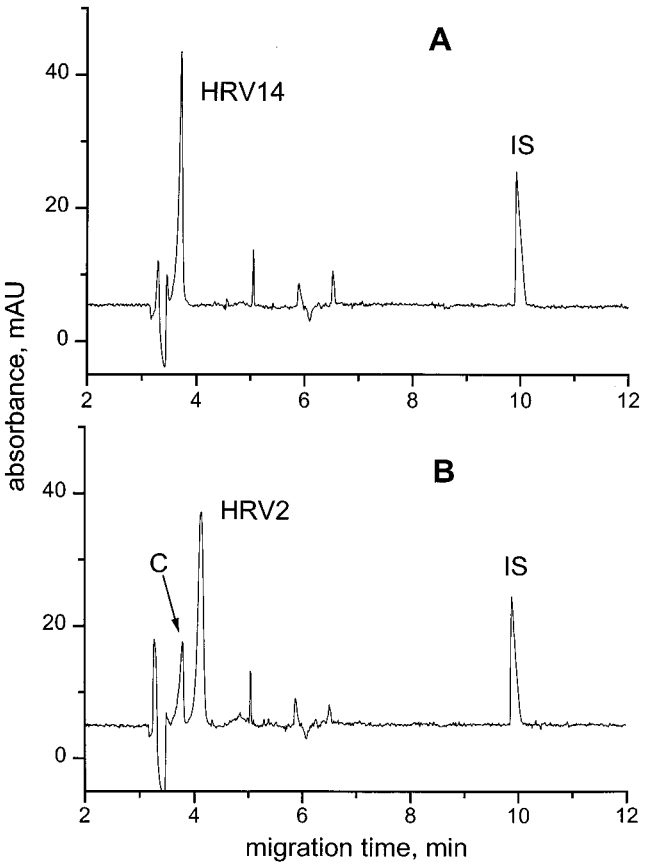

Figure 1. Electropherograms of preparations of purified HRV14 (A) and HRV2 (B). C, main contaminant (see text); IS, internal standard. BGE, modified RIPA buffer (100 mmol/L borate/boric acid, pH 8.3; 0.5% sodium deoxycholate, 0.05% SDS, 0.5% Triton X-100-R, all w/v). Conditions: untreated fused-silica capillary, 60.0 (51.5) cm length, 50 μm i.d.; voltage +25 kV. Detection at 205 nm. $T = 20$ °C.

tatively assigned to HRV14 (see Figure 1A). As all serotypes are routinely produced and purified by essentially the same methodology, it is likely that the same contamination is present in the HRV14 preparation. Comigration of the contaminant with HRV14 seriously impairs quantitation. To allow for better separation of HRV14 from this major contaminant, the BGE was modified.

Borate Buffer with Deoxycholate as Additive. Previous work with HRV2 had indicated that detergents were required as constituents of the BGE to reduce peak dispersion or loss of analyte due to adsorption.[5,6] As pointed out above, SDS is very useful in this respect; however, a major drawback is its denaturing potential. Fortunately enough, rhinoviruses are relatively stable in low concentrations of SDS; due to a particular hydrophobic binding pocket in the viral capsid which becomes occupied by the detergent, they are even stabilized against conformational changes occurring at low pH or at elevated temperature.[6,8,16] Nevertheless, subviral particles become disintegrated in SDS; we thus decided to investigate whether sodium deoxycholate alone as additive would lead to a better resolution of the analytes from the contaminants.

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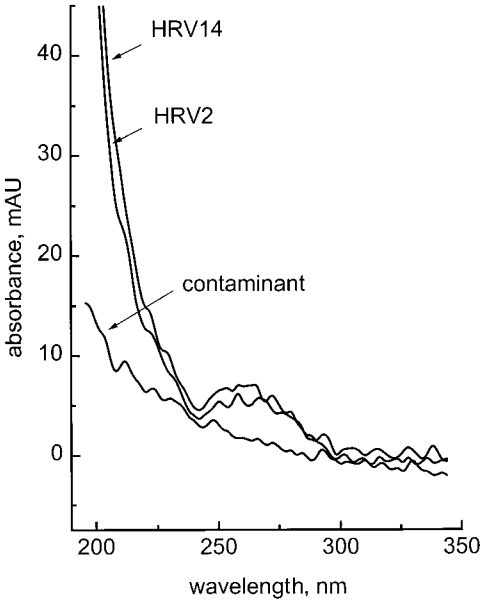

Figure 2. Comparison of the UV spectra obtained with the diode-array detector from the peak of HRV2, the peak tentatively assigned to HRV14, and the peak of an unidentified contaminant (C) present in the HRV2 preparations. See Figure 1 for reference to the peaks.

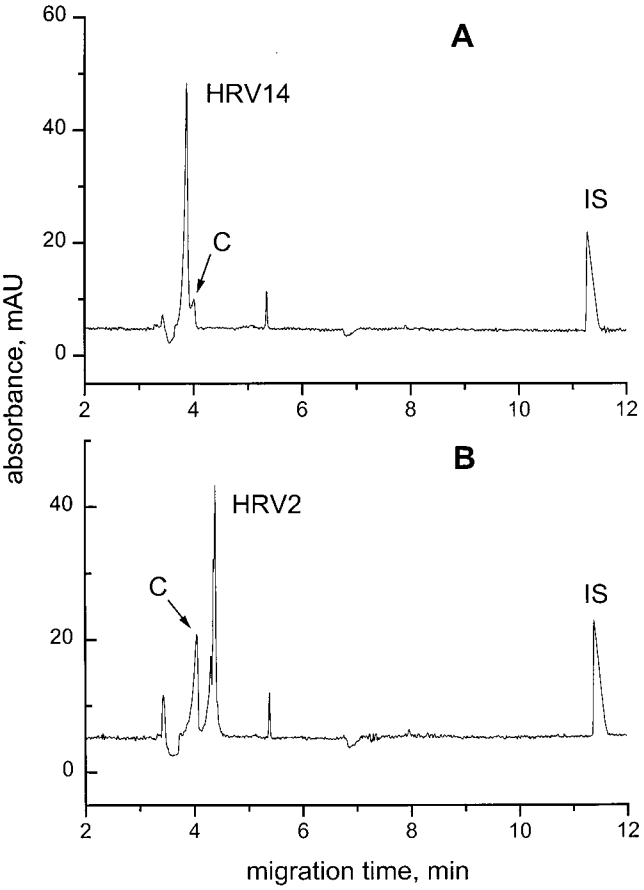

Figure 3. Electropherograms from the preparations of HRV14 (A) and HRV2 (B) in borate buffer (100 mmol/L borate/boric acid, pH 8.3) with deoxycholate (0.5%) as detergent. Other conditions were as in Figure 1.

For HRV2, separation from the main nonviral component was similar to that obtained in modified RIPA buffer (compare Figure 1 with Figure 3 and see also ref 6). In the case of HRV14, this

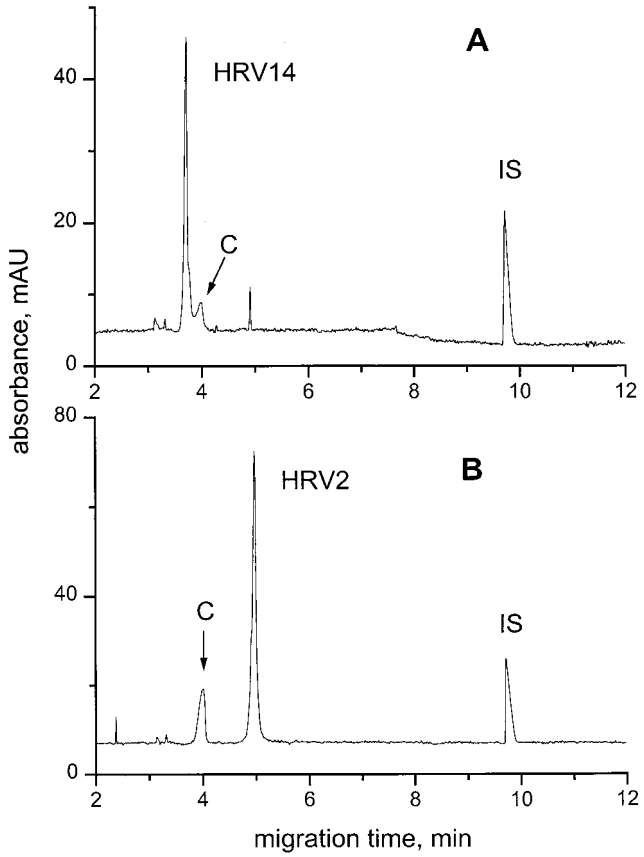

Figure 4. Electropherograms from the preparation of HRV14 (A) and HRV2 (B). BGE: borate buffer (100 mmol/L borate/boric acid, pH 8.3) with SDS (10 mmol/L = 0.26%) as detergent. Other conditions were as in Figure 1.

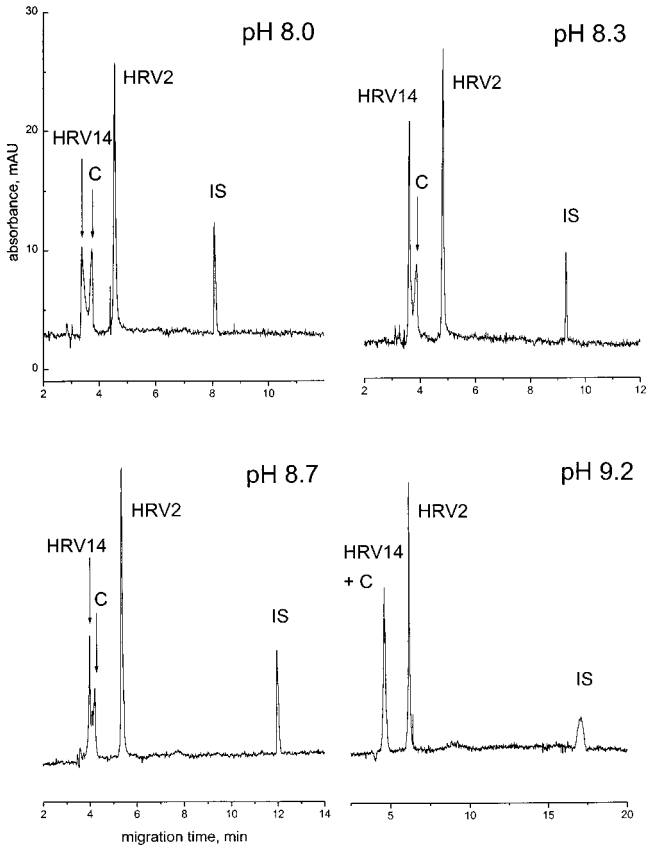

Figure 5. Influence of the pH on migration and peak shape of HRV14 and HRV2 in BGE containing 10 mmol/L SDS. HRV2 and HRV14 at ~0.2 mg/mL were mixed and injected into the capillary. Conditions were as in Figure 1.

particular BGE led to a resolution of the contaminant from the peak putatively assigned to HRV14 on the basis of the similarity of the spectrum with that of HRV2 (which was essentially as that shown in Figure 2). However, in this BGE, HRV2 gave rise to multiple peaks with fronting (Figure 3B). This phenomenon might suggest that the two serotypes bind the detergent to different extents.[17,18]

Borate Buffer with SDS as Additive. The two BGE systems investigated so far had the disadvantage of either insufficient resolution of HRV14 from contaminants (Figure 1A) or of HRV2 migrating as multiple peaks (Figure 3B). Therefore, we decided to again use SDS at 10 mmol/L as additive. From the electropherograms of the two virus serotypes shown in Figure 4 , it can be seen that addition of SDS had two favorable effects when compared to the other BGEs described above: (i) The peak of HRV14 is well separated from the zone of the EOF, where all noncharged potential impurities would migrate; this is seemingly caused by the larger negative charge conferred to the virus in this system due to binding of SDS. This effect improves the analysis, because it leads to a more rapid and more quantitative migration of the analyte out of the initial sample zone. (ii) The difference in electrophoretic mobility of both serotypes is larger. In addition, separation of the major impurity from the HRV14 peak is sufficient for the clear distinction. Both viral peaks have small shoulders, probably due to the microheterogeneity of the viruses possibly reflecting the presence of some copies of uncleaved VP0 (the precursor of VP2 and VP4) in the capsid.

Effect of pH. The pH of the BGE is one of the most important parameters in CE. Therefore, whether variation of the pH in the present BGE leads to an additional improvement in the electrophoretic properties of the viruses was investigated. However, the pH region, which can be covered for the analytes under consideration, is restricted. At pH <5.6, conformational changes of the virus occur which result in the formation of subviral particles.[9,19] As we avoided working in the critical pH range at the isoelectric points of native HRV2 and HRV14 (which are around 6.5[20,21]), we decided to vary the pH within 8 and 9.2.

The effect of the pH on the migration and peak shape of the two serotypes was investigated using a mixture of the viruses and is shown in Figure 5. Note that only for simplicity was a mixture taken, because there is no practical application that would require separation of serotypes; the analytical problem is directed to differentiation rather than to resolution. It was found that at all pH values between 8 and 9.2 the virus peaks can be fully differentiated. However, at pH 8.0 the peak of HRV14 is strongly tailing, seemingly due to adsorption. Increase of the pH to 8.7

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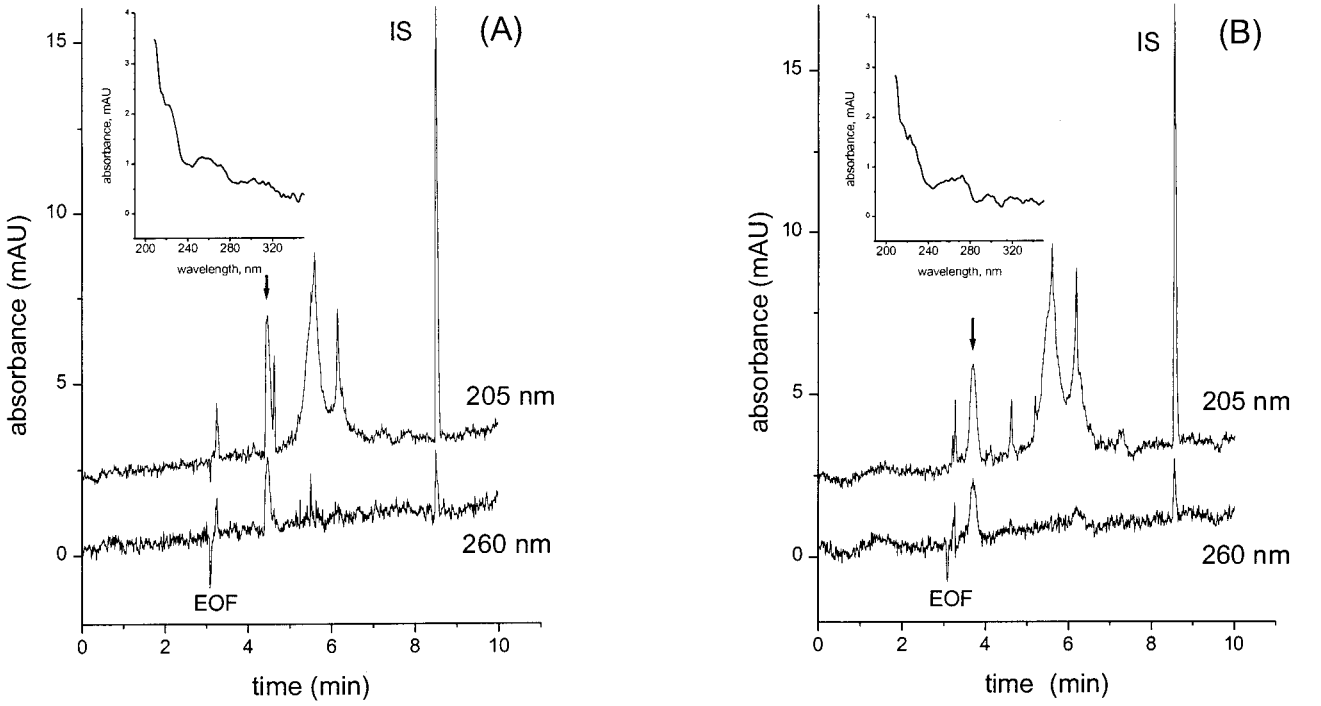

Figure 6. Electropherograms of the crude preparation of the HRV serotypes 16 (A) and 49 (B). Conditions as in Figure 4. The detector signal at 260 nm was amplified by a factor of 3. Insets show the spectra of the peaks indicated with an arrow as recorded with the diode-array detector. IS, internal standard.

led to comigration of the major impurity with HRV14, and at pH 9.2 both peaks fully overlap. Therefore, pH 8.3 appears to be most favorable for the unambiguous identification of the analytes.

Analysis of Crude Preparations of Serotypes HRV16 and HRV49. The method developed for the differentiation of the two serotypes 2 and 14 as described above was applied for the analysis of crude preparations of two other virus serotypes, namely, HRV16 and HRV49. In contrast to the samples of HRV2 and HRV14, these preparations contain the viruses at a much lower concentration, and a large number and amount of impurities are present. The electropherograms of these two preparations are shown in Figure 6. Many unresolved compounds, seemingly nonviral contaminants, appear in the time window between 5 and 7 min. These impurities were reproducibly found in such crude virus preparations and resulted in a very similar shape of the electropherograms outside the range where the virus serotypes 2 and 14 migrate. However, in the time window where these serotypes were recorded, only one main peak can be recognized in each crude preparation. It can be expected that the different serotypes exhibit a similar electrophoretic migration behavior due to the similarity of their protein shell (in fact, there was no reason to suppose a different electrophoretic behavior; this was why the present investigation was undertaken). Comparison of the migration times of the serotypes 16 and 49 with those from 2 and 14 (Figure 4) strongly suggests that these peaks indeed originate from virus.

Confirmation of this assumption is obtained from the spectral information of the peaks in the electropherograms. As pointed out, absorbance at 260 nm indicates the presence of RNA in the sample component, which is rather specific for viruses under the given circumstances. Indeed a small, but unambiguous local maximum at 260 nm is seen in the spectra of the two peaks (inset in Figure 6). Recording of the corresponding electropherograms at 260 nm shows clearly that the two peaks tentatively assigned as virus peaks give a signal at this detection wavelength that is clearly over the noise level. Even main contaminants recorded at 205 nm between 5 and 7 min lack a relevant signal at 260 nm.

Table 1. Mobilities of the Virus Serotypes and the Corresponding Electroosmotic Flow[a]

serotype	total virus mobility	EOF mobility	net virus mobility
HRV2	42.9	65.6	22.7
HRV14	56.0	65.6	9.6
HRV16	46.3	66.9	20.6
HRV49	55.7	66.7	11.0

[a] The mobilities (in 10^{-9} m^2 V^{-1} s^{-1}) were determined in borate buffer, 100 mmol/L, pH 8.3, 10 mmol/L SDS.

Differentiation of the Serotypes According to Their Mobility. The distinction of the serotypes by the electrophoretic assay presented here is based on their ionic mobilities. These data are given in Table 1. They range between 9.6 and 22.7 (10^{-9} m^2 V^{-1} s^{-1}), which means that the difference between the smallest and the largest mobility is remarkably large, namely, a factor of 2. All possible pairs of serotypes exhibit mobilities that differ at least by 15%, which is more than sufficient for a clear distinction, because it is a manifold of the standard deviation of their measurement (that is in ~1% only).

Infectivity Assay. To confirm the identity of a peak with infectious virus, we used off-line postcolumn detection by an infectivity assay for the viruses in the purified preparations. It was already mentioned that there is no reason to cut fractions from artificial mixtures of serotypes; in reality, the single species are present only. For the infectivity assay, four fractions were collected from each CZE run by programming the system to change the outlet vials every minute starting from 3.0 min after voltage

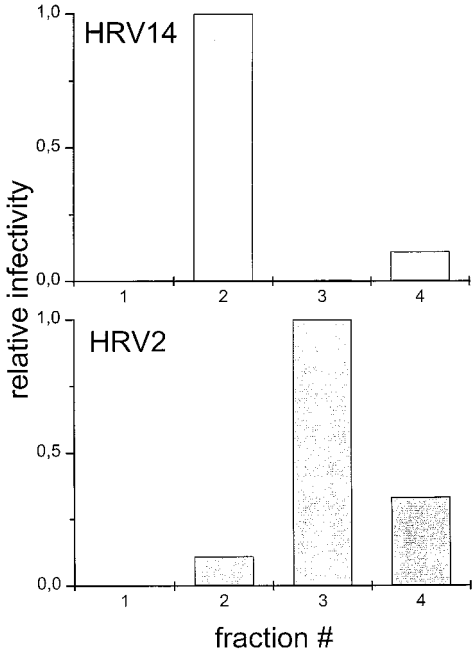

Figure 7. Distribution of infectious HRV2 and HRV14 over the four fractions collected at the outlet of the capillary during electrophoresis with the BGE containing 10 mmol/L SDS (Figure 4). Starting at 3 min after application of voltage, fractions were collected into 20 μL of BGE for 1 min each. Two microliters of each fraction were mixed with 150 μL of infection medium. A 100-μL aliquot of this solution (dilution step 0) were transferred to the first wells of a 96 well plate containing subconfluent HeLa cells. Serial 3-fold dilutions were added to the consecutive wells. Plates were incubated for 5 days, and infection was scored positive where cells were found to be lysed. The highest dilution of each fraction resulting in cell lysis was determined. For the sake of easy representation of the (relative) infectivity, this dilution was normalized to the maximum dilution causing cell lysis for any fraction collected from one run.

application. Taking into consideration the length of the capillary between the detector window and the outlet, it was calculated that these four fractions pass the detector between the following migration times: fraction 1, 2.6−3.4 min; fraction 2, 3.4−4.3 min; fraction 3, 4.3−5.2 min; fraction 4, 5.2−6.0 min. The peak of, for example, HRV14 should thus be collected in fraction 2 and the peak of HRV2 in fractions 3, respectively. Virus contained in the fractions was then determined by monitoring HeLa cell lysis as described in the Experimental Section.

As the fractions containing the largest number of viral particles lead to lysis of the cells at the highest dilution, the viral peak can be easily identified. As seen in Figure 7, viral analysis using the system containing 10 mmol/L SDS (see Figure 4) showed the highest virus concentration in fraction 3 for HRV14 whereas infectivity of HRV2 was highest in fraction 2, which corresponds well with the peaks seen in Figure 4. In addition, this result confirms the identity of the peaks eluting at 3.8 and at 4.5 min with infective HRV14 and HRV2, respectively. Virus was equally well determined in fractions obtained from separations carried out with the other BGE systems (data not shown).

CONCLUSIONS

In the present work we demonstrate clear differentiation of four HRV serotypes due to their distinct electrophoretic behavior. Detailed investigation of various BGEs led to a system that not only resulted in a maximum mobility difference of almost 13×10^{-9} m^{-2} V^{-1} s^{-1} but also allowed resolving the viruses from a major contaminant present in the preparations. This is important because it allows for an assessment of the purity of viral preparations and their quantification. Further, we demonstrate postcolumn detection of infectious virus by an infectivity assay based on lysis of HeLa cells. This assay, carried out with submicroliter fractions collected from the capillary outlet allowed confirmation that the peak putatively attributed to HRV14 was indeed corresponding to infectious virus. Assuming that the higher mobility was due to stronger binding of detergent, it might be investigated whether surface exposure of hydrophobic patches could be correlated with the electrophoretic migration behavior. As HRV14 and HRV16 belong to the major receptor group whereas HRV2 and HRV49 belong to the minor group, a correlation of mobility and receptor specificity appears to be improbable, which is clear from in pairs comparison of their mobilities (Table 1). It is thus likely that only the antigenic makeup of the virus determines the electrophoretic properties.

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