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Development and validation of a high-precision capillary electrophoresis method for main component assay of ragaglitazar

Søren Vig Lehmann[a,1], Akio Bent Bergholdt[b,*]

[a]Medicinal Chemistry IV, Health Care Discovery, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Maaloev, Denmark
[b]Protein Analysis, CMC Development, Novo Nordisk A/S, Hagedornsvej 1, DK-2820 Gentofte, Denmark

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Abstract

The ability of a developed capillary electrophoresis (CE) method for fast, efficient and reliable main component assay of ragaglitazar [NNC 61-0029/DRF(-)2725] has been demonstrated through documentation of the analytical performance and the results of a successful validation. The fast analysis time of around 1.2 min ensures a high analytical capacity, and the validation results show that the CE method is robust and gives reliable and precise results. The results from the validation of the CE method meet the acceptance criteria that are normally set for other main component assays such as high-performance liquid chromatography assays.
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Keywords: Validation; Method development; Pharmaceutical analysis; Ragaglitazar

1. Introduction

Ragaglitazar [NNC 61-0029/DRF(-)2725] shown in Fig. 1 is an efficacious, non-thiazolidinedione based dual peroxisome proliferator-activated receptor (PPAR) α,γ agonist that was selected as a drug candidate intended to restore insulin sensitivity and correct diabetic dyslipidaemia of patients with Type 2 diabetes [1–3].

As part of the standard drug development programme an analytical method for main component assay of NNC 61-0029 drug substance and drug product was required. High-performance liquid chromatography (HPLC) is typically the choice of analytical technique for this type of analysis because HPLC instruments are dominant in most pharmaceutical laboratories and staff are familiar with their use. However, especially in the later stages of drug

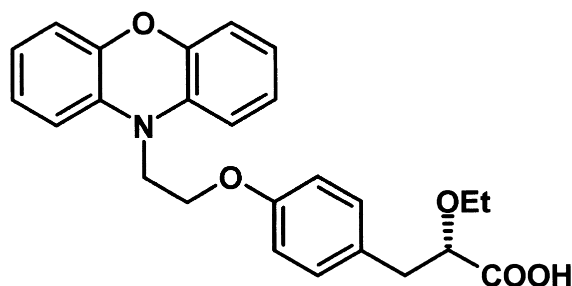

Fig. 1. Chemical structure of Novo Nordisk drug candidate NNC 61-0029.

*Corresponding author. Tel.: +45-44-434-544; fax: +45-44-438-400.
E-mail address: akio@novonordisk.com (A.B. Bergholdt).
[1]Present address: Analytical R&D, Mekos Laboratories A/S, Herredsvejen 2, DK-3400 Hillerød, Denmark.

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development where considerable analytical capacity is needed, the HPLC analysis time can be a critical factor. Furthermore, late eluting degradation products that are non-existant at the start of method development can cause peak disturbance or, in worst case, give unexpected results in later chromatograms. In addition, consumption of substantial amounts of mobile phases may afford a potential waste burden and therefore HPLC may not be the optimal selection of analytical methodology for high-throughput assaying of drugs.

Capillary electrophoresis (CE) is a relatively new analytical technique that is being implemented in the pharmaceutical industry [4,5]. CE has proved to be a powerful tool for the separation of various compounds due to the high efficiency and short analysis times that can be achieved [6–8]. In addition, the minimal consumption of sample and electrolyte with CE allow for rapid and inexpensive method development. Especially for the analysis of chiral compounds, the use of CE has decreased method development time due to the availability of various types of chiral selectors [9]. Thus, the application of CE for chiral analysis has been a significant improvement for the analysis of optical isomers where, traditionally, HPLC and gas chromatography (GC) have been used. Other areas where CE is able to challenge or supplement HPLC and GC are main component assay and purity testing that require high precision and sensitivity [4]. When CE was introduced, CE apparatus showed poor precision and sensitivity, but with technological improvements these issues have been addressed. Insufficient precision can be dealt with using an internal standard. Poor detector sensitivity has partly been solved by improvement of detection through increasing the detector path-length (i.e. Z-cell, bubble detector cell capillaries and high-sensitivity cells), improving detectors that can use detection wavelengths in the lower UV area, enhancing signal processing efficiency or using sample stacking [10–14]. Especially for main component assay of drugs in development, CE may become an important analysis tool because of its high analytical capacity, low solvent and sample consumptions and ease of analysis. Another important issue with CE is that there is no interference between analytical runs caused by late migrating impurities or degradation products when the capillary is flushed between each run. This is a potential problem often overlooked in HPLC analyses.

Development and validation of main component assays using CE for various pharmaceuticals have been reported in the literature and summarised recently by Altria [4]. In many of the applications, an internal standard was employed to improve precision. In particular, a CE assay for water-soluble acidic drugs using a high-pH (9.5) borate buffer and two internal standards gave good precision (RSD$<$1%) [15]. Another CE application based on a high-pH borate buffer, but without internal standard for the determination of enalapril maleate, afforded even better precision (RDS$=$0.62%, $n=10$) [16]. A recent example was presented by Furlanetto et al., who optimised and validated a simple and fast capillary zone electrophoresis (CZE) method for rufloxacin hydrochloride determination in tablets using an internal standard [17]. The method was validated according to International Conference on Harmonisation 3 guidelines (ICH3) documents Q2A and Q2B [18].

This paper presents the successful validation of a high precision CE assay method for fast and reliable main component assay of NNC 61-0029 drug substance, using a high-pH (9.3) borate buffer, quantification with external standard, and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) as peak shape improving buffer-additive. The method has sufficient injection repeatability for quantification with the use of external standard only.

2. Experimental

2.1. Materials

Heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) (minimum 90%) lot 67H1148 from Sigma (St. Louis, MO, USA) was used throughout the study. Then, 50 m*M* pH 9.3 borate buffer, 0.1 *M* and 1 *M* sodium hydroxide solutions for CE were purchased from Hewlett-Packard (Waldbronn, Germany). Water was obtained from a Millipore Milli-Q deioniser system (Millipore, Bedford, MA, USA).

The arginine salt of the drug candidate NNC 61-0029 [(*S*)-(−)-2-ethoxy-3{4-[2-(10-phenoxazin-

10-yl)-ethoxy]phenyl}propanoic acid] shown in Fig. 1 and potential synthesis impurities were prepared by the Chemical Development Department at Novo Nordisk (Copenhagen, Denmark).

2.2. CE instrumentation

For the CE assay, an $HP^{3D}CE$ capillary electrophoresis instrument (Hewlett-Packard) was used. The instrument was operated in the normal polarity mode at +30 kV/+40 μA and detection was performed on-column at 205 nm with 350 nm as reference wavelength. CE analysis was carried out using untreated fused-silica capillaries of 32.0 cm (23.5 cm effective length)×25 μm I.D. and an extended light path capillary with optical path-length of 125 μm. Capillaries were cut from 48.5 cm total length standard extended light path capillaries from Hewlett-Packard. The capillary was thermostatted at 30 °C. Samples were injected at the end of the capillary furthest away from the detector (long-end injection) using +50 mbar for 12 s. The HP Chemstation software (Hewlett-Packard) operated on a HP Kayak XA computer (Hewlett-Packard) was used for instrument control, data acquisition and data analysis. Electrophoretic data were collected at a rate of 10 Hz.

2.3. CE method preparations

The electrolyte was prepared by dissolving 2.0% (w/v) TM-β-CD in 50 m*M* pH 9.3 borate buffer and filtering through a 0.45 μm polypropylene filter (Whatman, Ann Arbor, MI, USA). Each new capillary was conditioned before use by rinsing for 20 min with 1 *M* sodium hydroxide. At the beginning of each working day, the capillary was conditioned by treatment with water for 5 min, 0.1 *M* sodium hydroxide for 5 min and water for 5 min. For every 15th run, the capillary was flushed with electrolyte for 5 min.

CE standard stock and test solutions were NNC61-0029 drug substance dissolved in acetonitrile–5 m*M* pH 9.3 borate buffer (5:95, v/v). The same NNC 61-0029arginine batch was used as sample and standard. Quantification was performed using external standard with one standard level at a nominal concentration of approximately 20 μg/ml and corrected peak areas. Corrected peak area is peak area divided by the corresponding migration time. Each determination corresponded to the average of two independent quantifications, and each quantification was performed by employing the results obtained through injecting the standard and test sample four times each in alternating sequence. The results were reported as the ratio of the corrected peak area of NNC 61-0029 in test sample and standard, as a percentage.

All concentrations were calculated as NNC 61-0029arginine salt concentrations. Separation efficiency in terms of theoretical plate counts, N, and symmetry factor, T, was calculated according to Ref. [19].

3. Results and discussion

3.1. CE method development

When developing new analytical methods based on chromatography or electrophoresis, parameters such as analysis time (t), theoretical plate number (N) and symmetry factor (T) are important for evaluation of the performance of the analytical method. Typically, an analytical method is optimised on the basis of these parameters before a validation is carried out, in order to obtain an overall good performance.

As reported in the literature, using a high-pH borate buffer and CE gave stable electroosmotic flow (EOF) conditions that allowed fast and efficient analysis of acidic NNC 61-0029 (Fig. 2a and Table 1). However, during method development it was discovered that addition of TM-β-CD to the run-buffer increased the theoretical plate number (N), improved the symmetry factor (T) and decreased the migration time (t) for NNC 61-0029, affording a faster and more efficient method, as shown in Fig. 2b and c and Table 1. With addition of 2.0% (w/v) TM-β-CD to the run-buffer, N reached 65 000 and T reached 0.73 while t was decreased to around 1 min which were considered sufficient for a fast and efficient CE main component assay. The optimal TM-β-CD concentration was not pursued any further.

A plausible explanation for the observed efficiency

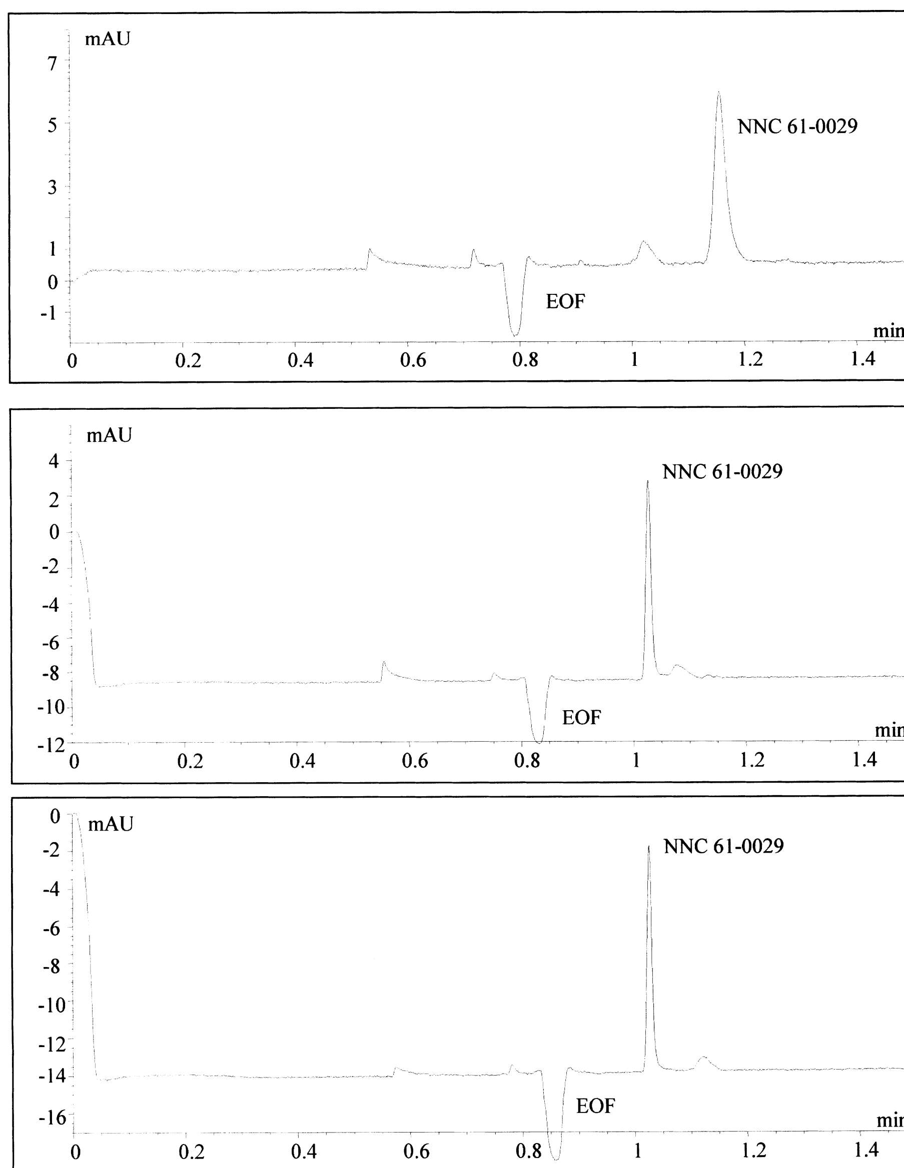

Fig. 2. Electropherograms showing the effect of addition of TM-β-CD to the run-buffer. Electropherograms (a) 0.0% (w/v), (b) 1.0% (w/v) and (c) 2.0% (w/v) TM-β-CD. Test sample concentration: 20 μg/ml. Run-buffer: 50 m*M* pH 9.3 borate buffer. Capillary dimensions: 32.0 cm(effective length 23.5 cm)×25 μm. Optical pathlength: 125 μm. Sample injection: 12.0 s at +50 mbar. Voltage: +30 kV. Current: +40 μA. Detection: UV at 205 nm. Temperature: 30 °C.

and migration time dependences on the TM-β-CD concentration is that neutral TM-β-CD interacts with negatively charged NNC 61-0029, which migrate in the opposite direction of EOF and away from the detector. The interaction causes a decrease in the migration velocity of NNC 61-0029 towards the sample inlet and thereby an increase in the effective mobility in the direction of the detector, thus leading to a faster migration time of NNC 61-0029. At the same time, when the analyte encounters neutral TM-β-CD in the capillary, NNC 61-0029 is concentrated to a narrower band that affords higher efficiency

Table 1
Dependence of the NNC 61-0029 migration time (t) and efficiency parameters (N and T) of the TM-β-CD concentration in run-buffer[a]

TM-β-CD (% w/v)	t (min)	N	T
0.0	1.15	16000	0.46
1.0	1.03	50000	0.70
2.0	1.02	65000	0.73

[a] Run-buffer: 50 mM pH 9.3 borate buffer.

(sometimes described as stacking). The increase in N, improves the precision of integration and therefore has a positive influence on method reproducibility. The stacking effect also increases the sensitivity of the method since the signal response is enhanced with higher concentration of TM-β-CD (Fig. 2). On the other hand, adding TM-β-CD to the run-buffer increases the viscosity of the electrolyte and results in a decrease in the EOF velocity and therefore an increase in migration time of EOF as shown in Fig. 2.

Minimal capillary dimensions (25 μm I.D., 32.0/23.5 cm total/effective length) and maximum field strength (+30 kV/32.0 cm) were chosen to match the instrumental limits in order to develop a method with the fastest possible analysis time. With the described electrolyte and instrumental settings, the total CE run-time was reduced to approximately 1.2 min. The time limiting step in the method became flushing between runs. However, investigations showed that flushing could be reduced to every 15th run without jeopardizing the stability and precision of the method, as illustrated in Fig. 3. Migration times for the 1st, 5th, 10th and 15th analysis without flushing in-between were very similar (Fig. 3). Thus, at pH 9.3 the strong EOF automatically refilled the capillary with electrolyte and obligatory flushing between runs could be omitted affording an even faster CE main component assay. The slightly longer migration time of the first injection (Fig. 3) could be related to the fact that the capillary was not fully conditioned with electrolyte.

3.2. CE validation results

Besides high efficiency and short analysis time, it is important, especially in the pharmaceutical laboratory, that the analytical method is robust and able to provide reliable data. To prove this, a validation according to generally accepted guidelines from institutional bodies such as the International Confer-

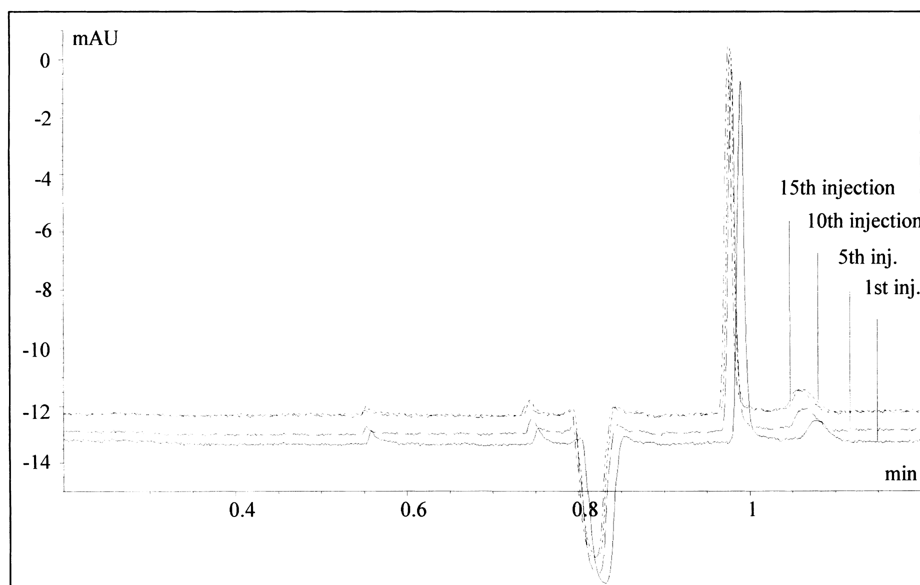

Fig. 3. Electropherograms of the 1st, 5th, 10th and 15th injection without flushing in-between runs. Test sample concentration: 20 μg/ml. Electrolyte: 2.0% (w/v) TM-β-CD in 50 mM pH 9.3 borate buffer. Other conditions as in Fig. 2.

ence on Harmonisation (ICH) or the Federal Drug Administration (FDA) is carried out. The validation result gives a valuable indication of the stability of the method and, together with the analytical performance, shows the methods overall performance.

The developed CE method was validated with respect to specificity, linearity, accuracy, injection repeatability, reproducibility and limit of detection.

3.2.1. Specificity

The specificity of the CE method was investigated by analysis of the individual synthesis intermediates, impurities and known degradation products, and by spiking a test sample with approximately 5% (w/w) of each component. In total, eight compounds together with the counter ion arginine were analysed individually and no interference with the main peak was observed. Furthermore, the results showed that the majority of the compounds migrated with the EOF. Thus, the method displayed excellent specificity. In Fig. 4 an electropherogram of a test sample spiked with the eight components can be observed.

In addition, a test sample with a placebo tablet matrix was constructed with the following analysis not revealing additional peaks in the electropherogram. Consequently, the CE method was applicable for analysis of NNC 61-0029 drug substance and drug product. However, the current validation was limited to the drug substance.

3.2.2. Linearity

The CE method was evaluated for linearity around the nominal concentration of 20 μg/ml. For this purpose, a calibration curve with five concentration levels in the range from 50 to 150% of the nominal concentration was constructed. Three injections were performed at each concentration level. The regression line was obtained by plotting the corrected peak area of the NNC 61-0029 peak versus the test sample concentration using the least squares method. The regression results are shown in Table 2.

The relationship between response and concentration was found to be linear in the investigated concentration interval with a correlation coefficient (r^2) of 0.99966. An examination of the corrected peak area residuals plotted against the test sample concentration did not reveal any sign of non-linearity (Fig. 5). The corrected peak area residual is equal to the observed average corrected peak area subtracted from the regression line predicted corrected peak area at a given concentration.

The results for linearity were satisfactory since correlation coefficients ≥0.999 normally are acceptable for HPLC validations. However, the origin was not included in the 95% confidence interval (C.I.) for single measurements. This was not regarded as a problem since drug substance sample concentrations of approximately 20 μg/ml were expected.

3.2.3. Accuracy

The accuracy of the CE method was assessed over

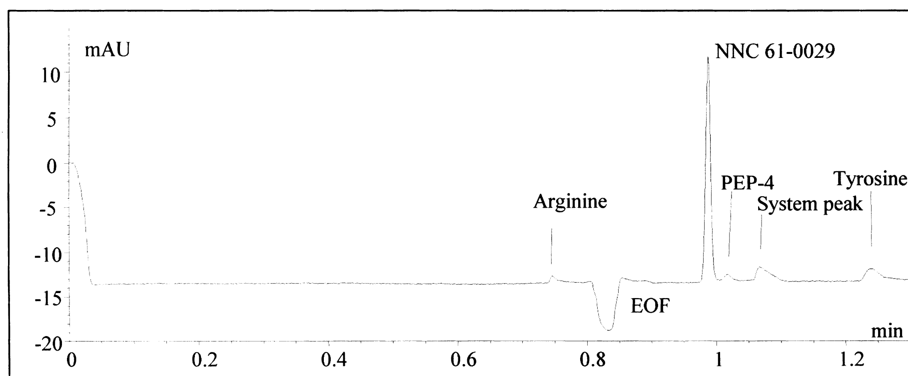

Fig. 4. Electropherogram displaying the specificity of the CE method. Test sample concentration: 40 μg/ml. Spiking level: about 5% (w/w) of each compound. Electrolyte: 2.0% (w/v) TM-β-CD in 50 m*M* pH 9.3 borate buffer. Other conditions as in Fig. 2.

Table 2
Linearity study parameters and regression results

Linearity	Value
Parameters	
Nominal concentration (μg/ml)	20
Concentration range studied (μg/ml)	10–30
Concentration range of nominal concentration (%)	50–150
Number of concentration levels	5
Number of injections at each concentration level	3
Regression results	
Correlation coefficient (r^2)	0.99966
Intercept	0.02927±0.01087
Slope	0.09995±0.00051
Origin included in 95% C.I.[a]	No
RSD at different concentration levels (%)	0.19 to 0.59

[a] 95% Confidence interval for single measurements.

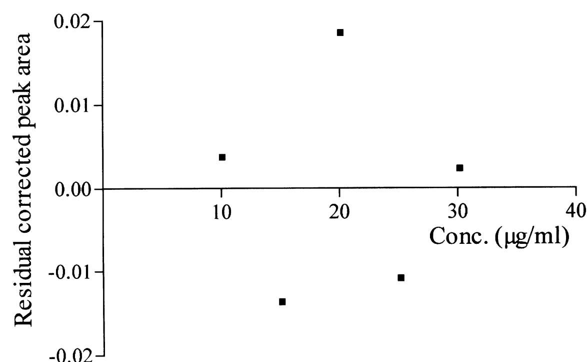

Fig. 5. Plot of residual corrected peak area vs. concentration of NNC 61-0029.

the same concentration range that was investigated in the linearity study (50–150%) by making three independent determinations at each concentration level. Mean recoveries with 95% confidence intervals (C.I.) are given in Table 3.

As shown in Table 3, satisfactory results were obtained for accuracy although a tendency towards overestimation and underestimation at 50% level and 150% level, respectively, were observed. Nevertheless, at the nominal concentration level (100%) that represented the concentration of expected samples, accuracy was more than acceptable.

Table 3
Accuracy results

Concentration levels (%)	Mean recovery±C.I.[a] (%)
50	103.0±3.4
100	100.1±1.6
150	98.7±0.6

[a] 95% Confidence interval for single measurements.

3.2.4. Precision

Precision of the CE assay was determined by measuring the injection repeatability and the reproducibility of the method.

3.2.4.1. Injection repeatability

In order to estimate the injection repeatability, five consecutive injections of the same test sample with a nominal concentration were performed. The obtained precision for injection repeatability calculated on the basis of corrected peak areas was 0.16% (RSD). This result was satisfactory, since generally a repeatability of less than 0.50% is acceptable in HPLC validations.

3.2.4.2. Reproducibility

The reproducibility of the CE method was explored by an inter-laboratory crossover study that included the following parameters: laboratory, instrument, day of analysis, capillary and analyst. The study was carried out as a complete statistical 2^3 factorial design with laboratory, instrument and day as one factor and capillary and analyst as the two other factors (Table 4). In total, eight independent

Table 4
Experimental design and results for evaluation of the reproducibility at the nominal concentration (20 μg/ml)

Determination number	Factor			Recovery (%)
	Capillary	Laboratory/Instrument/Day	Analyst	
1	A	Lab. 634/CE-1/29 March 2002	Operator 1	98.53
2	A	Lab. 634/CE-1/29 March 2002	Operator 2	98.92
3	A	Lab. 456/CE-4/09 April 2002	Operator 1	99.34
4	A	Lab. 456/CE-4/09 April 2002	Operator 2	99.57
5	B	Lab. 634/CE-1/29 March 2002	Operator 1	99.15
6	B	Lab. 634/CE-1/29 March 2002	Operator 2	99.93
7	B	Lab. 456/CE-4/09 April 2002	Operator 1	99.50
8	B	Lab. 456/CE-4/09 April 2002	Operator 2	99.41

determinations were performed. The results for the individual determinations as the ratio of the corrected peak area of NNC 61-0029 in test sample and standard (as a percentage) are tabulated in Table 4.

As shown in Table 5, the reproducibility study gave a mean value of 99.29% and an RSD between results of 0.43%. For single measurements, the 95% confidence interval was ±1.1% (Table 5). The obtained precision for reproducibility was excellent since in general 1–2% (RSD) is regarded as satisfactory for main component assays. Factors that certainly contributed to the remarkably high precision were the high pH borate buffer, the long injection time, the high efficiency and the short analysis time. The high pH borate buffer had sufficient buffer capacity to provide stable conditions for EOF and migration of NNC 61-0029, whereas the long injection time together with the adequately controlled temperature afforded low variance in injection volume. In addition, the high efficiency reduced integration errors and the short analysis time delayed buffer depletion effects resulting in a high precision CE method.

3.2.5. Limit of detection

The limit of detection (LOD) for NNC 61-0029 was estimated to be 0.20 μg/ml by diluting a test sample solution until the signal-to-noise ratio reached approximately three.

The obtained LOD indicates that the sensitivity of the CE method was limited. This was nevertheless not a problem, since the CE method was developed for assaying drug substance and drug product in routine samples where the content of drug candidate was well above the LOD.

In summary, the validation study shows that, besides excellent analytical performance, the CE method provides satisfactory validation data that are similar to those normally accepted for other main component assays such as HPLC and GC assays.

Table 5
Reproducibility results calculated using the values from Table 4

Reproducibility	Value
Mean recovery (%)	99.29
RSD (%)	0.43
C.I.[a] (%)	1.10

[a] 95% Confidence interval for single measurements.

4. Conclusions

The ability of the developed CE method to provide a fast, efficient and reliable main component assay of ragaglitazar [NNC 61-0029/DRF(-)2725] has been demonstrated through documentation of the analytical performance and the results of a successful validation. The total analysis time of around 1.2 min ensures a high analytical capacity while the validation results show that this CE method is robust and provides reliable and precise results. The obtained validation data are similar to those normally accepted for other main component assays such as HPLC and GC assays.

In addition, there is an environmental benefit of using CE because of low solvent and sample consumptions. Moreover, CE's ease of analysis makes rapid and inexpensive method development possible. Therefore, for main component assays, CE may be

an excellent analysis tool for high-throughput assaying of drugs.

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