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Separation and simultaneous determination of nalidixic acid, hydroxynalidixic acid and carboxynalidixic acid in serum and urine by micellar electrokinetic capillary chromatography

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Abstract

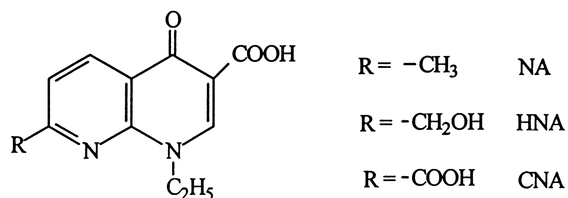
Separation in capillary electrophoresis is governed by various factors, including buffer type, buffer concentration, pH, temperature, voltage and micelles. Through proper adjustment of these parameters, nalidixic acid and its two major metabolites, 7-hydroxynalidixic and 7-carboxynalidixic, could be separated by micellar electrokinetic capillary chromatography using an electrophoretic electrolyte consisting of 50 m*M* borate buffer (pH 9) containing 25 m*M* sodium dodecyl sulphate and 10% acetonitrile. A linear relationship between concentration and peak area for each compound was obtained in the concentration range 0.15–100 μg ml^{-1}, with a correlation coefficient greater than 0.999 and detection limits in the 0.2–0.7 ng ml^{-1} range. Intra- and inter-day precision values of about 0.8–1.2% RSD (n=11) and 1.3–2.0% RSD (n=30), respectively, were obtained. The method has been applied to the analysis of nalidixic acid and its two major metabolites in serum and urine with limits of sensitivity lower than 0.8 ng ml^{-1}. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Nalidixic acid; Hydroxynalidixic acid; Carboxynalidixic acid

1. Introduction

Nalidixic acid (NA: 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) is an antibacterial agent that has been extensively used in the treatment of acute and chronic urinary tract infections caused by susceptible gram-negative organisms [1].

NA is partially metabolized in the liver to 7-hydroxynalidixic acid (HNA). The drug is also partially metabolized to the dicarboxylic derivative, 7-carboxynalidixic acid (CNA); there is some evidence suggesting that this metabolite is formed in the kidney. HNA possesses an antibacterial spectrum similar to that of NA and represents 30% of the antibacterial activity in the blood and 80–85% of the antibacterial activity in urine. CNA is reported as being inactive [2].



Several different methods have been used to determine NA, including ultraviolet spectrometry [3],

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fluorometry [4,5] and polarography [6,7], although all these methods have several disadvantages: they are non-specific towards the metabolites and give relatively high blank values and non-linear calibration curves. Two high-performance liquid chromatography (HPLC) methods have been reported; the first makes use of an ion-exchange column [8] and in the second [9] NA is methylated and chromatographed on a reversed-phase system. In addition, two gas–liquid chromatographic methods [10,11] have been described, but their application is limited to the determination of NA alone in tablets or in plasma.

Mixtures of NA and HNA in plasma [12] and NA, HNA and CNA in plasma and urine [13] have been determined using HPLC methods but prior extraction procedures are recommended in order to avoid endogenous interferents.

Micellar electrokinetic capillary chromatography (MEKC) [14], one of the most important modifications of capillary electrophoresis (CE), has proved to be an excellent alternative to HPLC in drug analysis. MEKC procedures exhibit greater resolution than HPLC and have the same order of repeatability. MEKC is faster and less costly to operate than HPLC, and, in some instances, has replaced HPLC as the analytical method of choice.

The purpose of this work was to investigate the separation of NA, HNA and CNA by CE, the two routes studied being the pH of the carrier electrolyte and the presence of surfactants. The last approach, where the separation of NA and its two metabolites is carried out using a borate buffer modified with sodium dodecyl sulphate (SDS) and acetonitrile, gave the best resolution.

The proposed MEKC method was used to determine NA, HNA and CNA in serum and urine samples. Further, this method may be useful for therapeutic drug monitoring in hospitals because of the ability of automation and short analysis time.

2. Experimental

2.1. Apparatus

Electrophoresis was carried out on a P/ACE System 5500 (Beckman Instruments, Palo Alto, CA, USA) equipped with a diode-array detector, an automatic injector, a fluid-cooled cartridge and a System Gold data station. Separations were performed in an uncoated fused silica capillary (Beckman Instruments) [57 cm (50 cm to the detector) $\times$75 μm I.D. $\times$ 375 μm O.D.).

A Unicam UV–Vis spectrophotometer (Unicam Limited, Cambridge, UK) was used for recording absorbance spectra of NA, HNA and CNA.

2.2. CE conditions

The high-voltage power supply was set to 15 kV (normal polarity), resulting in a typical current of 32–34 μA. Sample introduction was made at the anode side using the pressure option (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa) for 2–10 s and the drugs were detected at 246 nm. The temperature was kept constant at 25°C. The capillary was flushed with running buffer for at least 4 min between analyses. Before the first use, the capillary was conditioned by flushing for 5 min with freshly prepared 0.1 *M* sodium hydroxide and rinsing with the buffer for 2 min and then equilibrating with the buffer for 10 min, applying the separation voltage of 15 kV. The capillary inlet and outlet vials were replenished after every 10 injections. Unless otherwise stated, a 50 m*M* borate buffer of pH 9.0 containing SDS (25 m*M*) and acetonitrile (10%, v/v) was used as the running buffer.

2.3. Reagents and solutions

All solutions were prepared using 18 MΩ deionized water using a Milli-Q water-purification system (Millipore). NA was purchased from Sigma and HNA and CNA both from Sanofi-Wintrop.

Stock standard solutions of NA, HNA and CNA were prepared by dissolving 25.0 mg of the corresponding compound in 0.01 *M* sodium hydroxide and diluting with deionized water up to 250 ml in a calibrated flask. These solutions showed a pH of about 8 and remained stable for at least two weeks if kept refrigerated. Working solutions of lower concentrations were freshly prepared by dilution of the stock solution with deionized water.

2.4. Determination of NA, HNA and CNA in serum and urine

The samples of serum or urine (100 μl) were acidified with 100 μl of 0.1 *M* hydrochloric acid and subsequently extracted with 2 ml of chloroform. After mixing with a Vortex mixer for 1 min and centrifugation at 2500 *g* for 2 min, the chloroform layer was transferred to another centrifuge tube and evaporated to dryness. The residue was dissolved in 1 ml of the electrophoretic electrolyte.

3. Results and discussion

3.1. Method development

The method was started with 50 m*M* borate buffer (pH 10) as electrolyte. At pH 10, NA, HNA and CNA should be negatively charged. Because the electroosmotic migration velocity is greater than the electrophoretic migration velocity of these three compounds, a positive potential should be applied across the capillary and the solutes should migrate from the source to the detector at a velocity lower than that of the electroosmotic flow.

The migration times of NA, HNA and CNA are pH-dependent. The best pH range for the separation of the three compounds proved to be between 8.5 and 10.5 and this range was studied closely. A borate buffer of 50 m*M* at pH 9 was chosen for further experiments. The separation of HNA from NA and/or CNA was very good, although the pair NA–CNA was poorly resolved.

To resolve NA, HNA and CNA, a MEKC method was developed in which anionic and cationic surfactants [SDS, sodium cholate (SC), sodium taurocholate (STC) and *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB)] in 50 m*M* borate buffer (pH 9) were studied.

CTAB was not suitable as a micelle modifier because the migration times were very long (more than 40 min) and the resolution of the peaks of NA and CNA was very poor. SC and STC were also unsuitable because of the peak tailing observed for NA. When SDS was added to the running electrolyte, a clear improvement of the separation was observed. Increasing SDS concentration had no effect on the migration order of the compounds although the migration times became longer and peak separation improved greatly; however, a SDS concentration greater than 30 m*M* is not recommended because the HNA peak splits into two poorly resolved peaks. A SDS concentration in the range 20–30 m*M* is the best compromise for achieving good separation of the three compounds.

Selectivity may also be manipulated by varying the aqueous organic modifier [15] in addition to the surfactants species, concentrations and pH. The effect of adding up to 15% acetonitrile on solute separation was tested. The migration times increased with the increasing acetonitrile contents. The electrophoretic velocities of the anionic solutes did not alter significantly but the migration velocity, which is the sum of the electrophoretic and electroosmotic velocities, was reduced considerably because of the decreased electroosmotic velocity in the experimental conditions. Hence, 10% (v/v) acetonitrile was added to the electrophoretic electrolyte in all subsequent studies in order to improve peak shape.

The influence of temperature on the separation of NA, HNA and CNA was also tested. To obtain reproducible results, it is very important to keep the temperature constant during the analysis, for example by using capillary cooling, as in this study. The separation was always carried out at 25°C.

After adjusting buffer and surfactant concentration, pH, organic solvent, temperature and voltage, we chose 25 m*M* SDS in 50 m*M* borate buffer pH 9 containing 10% (v/v) acetonitrile (15 kV, 25°C) as electrolyte for the quantification studies.

A study was performed to determine the degree of trace enrichment at various injection times and any resulting a sacrifice in resolution. It was possible to perform pressure injections of up to 18 s with no loss in resolution and with fair degree of linearity of peak area versus injection time (analyte quantity). It was also observed that injection times longer than 20 s resulted in poor peak shapes because of disturbance of the sample–buffer interface zone.

3.2. Calibration graph, detection limit and reproducibility

Calibration graphs were obtained by injecting standard solutions of the analytes in the concen-

tration range 0.15–100 μg ml^{-1}. Each point of the calibration graph corresponded to the mean value obtained from three independent measurements of peak area. The detection limits were estimated through the IUPAC model modified for chromatography [16]. The corresponding regression equations and other characteristic parameter are shown in Table 1.

The reproducibility of the method was studied by measuring the migration times, peak area and peak heights of 11 consecutive runs. The relative standard deviation (RSD) of the migration time of the three analytes was within the range 0.1–0.6%. Both peak area and peak heights were reproducible with an RSD of less than 1.2%. When the same sample was run 30 times over an eight-day period, the RSDs were 1.4%, 1.3% and 2.0% for NA, HNA and CNA, respectively.

3.3. Applications

The MEKC assay is characterized by long-term stability and reproducibility. To demonstrate the usefulness of the procedure for the determination of NA, HNA and CNA, human serum and urine were analysed for the presence of these drugs.

The calibration curves were constructed by analysing seven plasma and eight urine samples spiked with NA, HNA and CNA. The concentration ranges studied in plasma (0.1–20 μg ml^{-1}) and urine (1–100 μg ml^{-1}) were chosen to correspond to the expected concentrations in biological fluids after a single therapeutic administration of NA in humans. Quantification was carried out using either the external or internal standard calibration methods. Pipemidic acid was chosen as the internal standard since it is extracted from acidified solutions, and can be detected at 246 nm. It also has the required electrophoretic properties. The most representative data concerning to the determination of each drug using the internal standard calibration method are summarize in Table 2.

Fig. 1 shows an electropherogram of a serum sample spiked with NA, HNA and CNA and the internal standard and then treated following the procedure described in Section 2.4.

The relative recoveries obtained in the analysis of three different samples of serum and urine are indicated in Table 3. As can be seen, there were no significant differences between the results obtained using the external and internal standard calibration methods.

Peak confirmation and peak purity evaluation were achieved by comparison of the migration time of each compound with that of the standard solution and by comparison of the absorption spectra obtained from the electropherograms of these compounds with those of the standard in water.

4. Conclusions

This is the first report dealing with the determination of NA, HNA and CNA using capillary

Table 1
Figures of merit of MEKC method for determination of NA, HNA and CNA[a]

Compound	$y=a+bx$	$S_{y/x}$	r^2	D.L.	S.E.
NA	$a=0.009482\pm0.014970$ $b=0.051514\pm0.000680$	0.020348	0.9996	0.6	699 000
HNA	$a=0.078803\pm0.031760$ $b=0.143656\pm0.001552$	0.031564	0.9997	0.2	293 000
CNA	$a=0.020709\pm0.009653$ $b=0.049648\pm0.000422$	0.066950	0.9998	0.7	567 000

[a] b is the slope of the regression lines fitted to the calibration data set±standard deviation; a is the intercept of the regression lines fitted to the calibration data set±standard deviation; r^2 is the correlation coefficient of the calibration graph; $S_{y./x}$ the standard deviation from the calibration graph; D.L. is the detection limit in ng ml^{-1} (signal-to-noise ratio = 3) and S.E. is the separation efficiency (number of theoretical plates per meter).

Table 2
Linear standard curves for the determination of NA, HNA and CNA in serum and urine samples

Biological fluid	Compound	No. of points	Slope ±SD	Intercept ±SD	r^2	D.L. (ng ml^{-1})
Serum	NA	7	0.05202 ±0.0007	0.00898 ±0.0015	0.995	0.5
	HNA	7	0.14978 ±0.00163	0.07622 ±0.0018	0.997	0.3
	CNA	7	0.05047 ±0.00082	0.02261 ±0.0017	0.996	0.6
Urine	NA	9	0.05153 ±0.00084	0.00874 ±0.0019	0.995	0.6
	HNA	9	0.14386 ±0.00164	0.07448 ±0.00334	0.998	0.3
	CNA	9	0.04958 ±0.00014	0.02201 ±0.00113	0.994	0.7

electrophoresis. The method is shown to have good reproducibility, a wide linear response range and low mass detection limit.

The development of a MEKC-based method for the determination of NA and its two major metabolites in serum and urine highlights the ability of CE to provide a rapid assay with a sensitivity limit of about 0.5 ng ml^{-1}. The data obtained confirm the possibility of using MEKC for pharmacokinetic studies of NA in humans. Unfortunately, the method could not be tested using blood serum and urine samples from individuals taking NA because such samples were not available.

Acknowledgements

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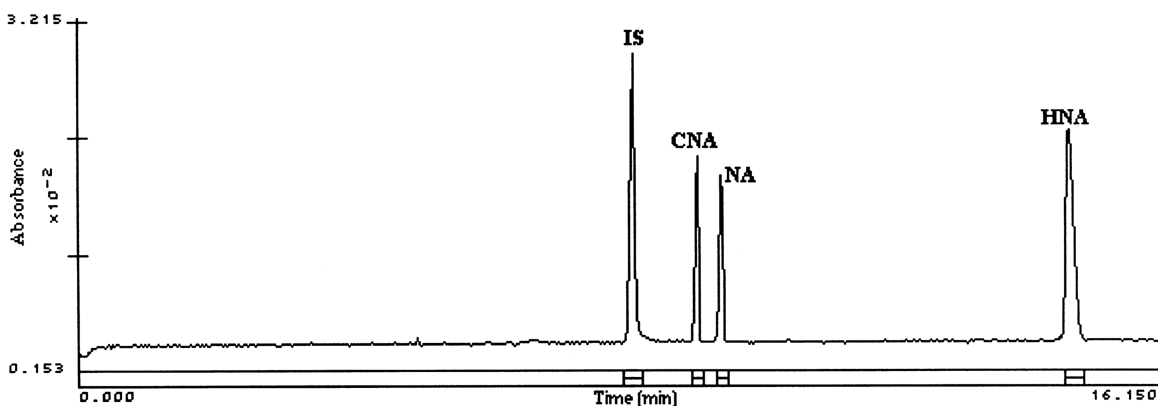

Fig. 1. MEKC separation of nalidixic, 7-hydroxynalidixic and 7-carboxynalidixic acids in serum. Electrolyte solution: 50 m*M* borate buffer (pH 9), 25 m*M* SDS and 10% (v/v) acetonitrile.Concentrations (μg ml^{-1}): NA, 1.3; HNA, 1.2; CNA, 1.2; I.S., 4.0.

Table 3
Recoveries of NA, HNA and CNA from serum and urine

Biological fluid	NA			HNA			CNA		
	Added[a] (μg/ml)	Mean recovery (%)	RSD (%)	Added (μg/ml)	Mean recovery (%)	RSD (%)	Added (μg/ml)	Mean recovery (%)	RSD (%)
Urine 1	0.6 (4)	96.4[b]	0.9	0.6 (4)	94.2[b]	0.4	0.6 (4)	106.7[b]	1.0
		98.6[c]	0.6		95.2[c]	0.9		107.5[c]	1.1
Urine 2	1.2 (4)	96.6[b]	0.7	1.2 (4)	98.8[b]	0.8	1.2 (4)	96.8[b]	0.4
		97.0[c]	0.3		99.7[c]	0.6		97.3[c]	0.9
Urine 3	12.0 (4)	98.9[b]	1.4	12.0 (4)	96.0[b]	0.7	12.0 (4)	99.4[b]	1.3
		98.6[c]	0.4		98.6[c]	1.1		101.2[c]	0.5
Serum 1	0.6 (4)	92.7[b]	0.8	0.6 (4)	97.2[b]	1.2	0.6 (4)	93.2[b]	0.9
		96.6[c]	0.3		92.1[c]	0.8		106.1[c]	1.0
Serum 2	1.2 (4)	97.2[b]	0.7	1.2 (4)	94.2[b]	1.8	1.2 (4)	95.9[b]	0.9
		96.9[c]	0.4		94.0[c]	1.0		93.1[c]	0.5
Serum 3	12.0 (4)	100.4[b]	0.2	12.0 (4)	100.6[b]	0.3	12.0 (4)	101.8[b]	0.2
		98.5[c]	0.6		98.6[c]	0.4		100.0[c]	0.6

[a] Number of samples are in parentheses.
[b] Values obtained using the external standard calibration method.
[c] Values obtained using the internal standard calibration method.

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