Development and Validation of a High-Performance Liquid Chromatography Method for the Evaluation of Niflumic Acid Cross-Reactivity of Two Commercial Immunoassays for Cannabinoids in Urine

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Abstract

Niflumic acid is a nonsteroidal, anti-inflammatory drug widely prescribed in Greece. We recently noticed that this drug cross-reacts for cannabinoids in a kinetic interaction of microparticles in a solution (KIMS) immunoassay method but does not in an enzyme multiplied immunoassay technique (EMIT) immunoassay method. The objective of the study was to develop and validate a high-performance liquid chromatographic method in order to evaluate niflumic acid cross-reactivity in two commercial immunoassays for cannabinoids in urine, both in niflumic acid standards as well as in urine specimens obtained from subjects receiving niflumic acid. Urine niflumic acid standards were prepared in drug-free urine at 13 concentrations ranging from 1.25 to 1000 µg/mL. The standards gave presumptive positive cannabinoids results when analyzed by the KIMS immunoassay method when the concentration was above 2.5 µg/mL. None of the prepared standards gave a false-positive cannabinoid result when analyzed by the EMIT immunoassay method. By applying a 50 ng/mL cutoff for cannabinoids in these assays, all 55 urine specimens collected from the 5 subjects who participated gave negative results by the EMIT and false-positive results by the KIMS immunoassay method. It is concluded that KIMS is more prone to cross-reactions by niflumic acid compared to EMIT. Therefore, all positive screening tests for cannabinoids obtained by KIMS should be confirmed by another technique.

Introduction

Immunoassay procedures, with radioimmunoassay being the first one in 1976 (1), are widely used to screen urine samples for recent marijuana use by analyzing the samples for 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (11-nor-Δ^9-THC-9-COOH), which is the major urinary metabolite of Δ^9-tetrahydrocannabinol (Δ^9-THC).

The enzyme multiplied immunoassay technique (EMIT) methodology utilizes monoclonal antibody reactive to Δ^9-THC, labeled with glucose-6-phosphate dehydrogenase.

The kinetic interaction of microparticles in a solution (KIMS) methodology utilizes antibody-bound microparticles.

Although immunoassay techniques are widely used in screening, they are prone to cross-reactions and false-positive results (2), and therefore, all positive screening results should be confirmed by a more specific analytical method.

Niflumic acid [2-((3-(trifluoromethyl)phenyl)amino)-3-pyridinecarboxylic acid] (Figure 1) is widely prescribed in Greece. It is a nonsteroidal, anti-inflammatory drug that acts by inhibiting isoforms of cyclo-oxygenase 1 and 2.

Niflumic acid is readily absorbed after oral administration. It is metabolized by hydroxylation and glucuronic acid conjugation. About 40% of a dose is excreted in the urine within 48 h, and about 30% is eliminated in the feces within 72 h. Peak-plasma concentrations are found within 2–3 h post-dose in subjects following a 250-mg dose (3).

Cross-reactivity of various compounds in immunoassay screening for cannabinoids has been examined in the past (4–7). Nevertheless, to our knowledge, there is no previous report of niflumic acid cross-reactions in immunoassay screening for cannabinoids.

The package insert for the analyzer utilizing the EMIT methodology provides no information about niflumic acid

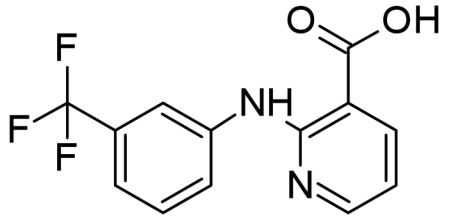

Figure 1. Chemical structure of niflumic acid.

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cross-reactivity in the cannabinoids assay.

On the other hand, the package insert for the analyzer utilizing the KIMS methodology mentions that for the 50 ng/mL cutoff, the cross-reactivity for niflumic acid at a concentration of 678 ng/mL is 7%.

We recently screened two individuals for cannabinoids by KIMS, and they initially tested positive. Upon confirmation by high-performance liquid chromatography (HPLC), no cannabinoids were detected. Subsequently, the individuals were questioned about the medication they had recently received, and in both cases niflumic acid was involved. In both cases, the drug was detected in the urine samples by HPLC.

Because of these two alarming incidences, the concern about niflumic acid cross-reactivity in the cannabinoids immunoassay and the wide prescription of the drug in Greece, the following study was performed.

Materials and Methods

Niflumic acid was purchased in its pharmaceutical formulation commercially available in Greece (NIFLAMOL/Bristol Myers Squibb, New York, NY).

Standard solutions of niflumic acid were prepared in drug-free urine at the following concentrations: 1.25, 2.5, 5, 10, 30, 50, 70, 100, 150, 200, 500, 700, and 1000 µg/mL. The standard solutions were screened in duplicate for cannabinoids, as unknown samples, both by the KIMS as well as the EMIT immunoassay. The results were evaluated by applying a 50 ng/mL cutoff for cannabinoids. Assay calibration and analysis of control samples were performed as specified by each manufacturer.

For the needs of the second part of the study, five healthy volunteers (age 25–35 years) received niflumic acid (250 mg × 3) for a period of eight days. All participants signed an informed consent form. Their urine samples were collected each morning, beginning on the second day and ending four days after the discontinuation of niflumic acid (Table I). A total of 55 samples were collected (11 samples from each participant). The samples were kept frozen until analysis. They were screened in duplicate both by the EMIT and the KIMS immunoassay and then analyzed by HPLC.

For each day of the experiment, the mean EMIT and KIMS rate were calculated based on the screening result of the urine samples provided for that day from the five participants.

Table I. Therapeutic Regimen and Urine Samples Tested for Immunoreactivity By KIMS and EMIT Cannabinoids Assay and Niflumic Acid Concentration as Determined by HPLC*

Urine Sample Code	Day of Experiment	KIMS Result Cannabinoids (ng/mL)	EMIT Result Cannabinoids (ng/mL)	Niflumic Acid Concentration by HPLC (µg/mL)
	Day 1: 250 mg niflumic acid × 3 times per day			
1	Day 2: 250 mg niflumic acid × 3 times per day	1694 (positive)	< 20 (low control)	50.5
2	Day 3: 250 mg niflumic acid × 3 times per day	1574 (positive)	< 20 (low control)	32.4
3	Day 4: 250 mg niflumic acid × 3 times per day	1298 (positive)	< 20 (low control)	20.3
4	Day 5: 250 mg niflumic acid × 3 times per day	1273 (positive)	< 20 (low control)	17.0
5	Day 6: 250 mg niflumic acid × 3 times per day	1252 (positive)	< 20 (low control)	15.5
6	Day 7: 250 mg niflumic acid × 3 times per day	1231 (positive)	< 20 (low control)	14.4
7	Day 8: 250 mg niflumic acid × 3 times per day	1208 (positive)	< 20 (low control)	13.6
8	Day 9: No medication	1150 (positive)	< 20 (low control)	12.7
9	Day 10: No medication	222 (positive)	< 20 (low control)	8.9
10	Day 11: No medication	151 (positive)	< 20 (low control)	3.4
11	Day 12: No medication	63 (positive)	< 20 (low control)	1.7

* Each column represents mean KIMS result, mean EMIT result, and mean niflumic acid concentration, respectively, as calculated from five different urine samples collected from the five participants for each urine sample number (for each day).

Table II. Calibration and Sensitivity Data for Niflumic Acid in Standard Solutions and Urine Samples

Analyte	Regression Data*	*R*	LOD (µg/mL)	LOQ (µg/mL)
Standards				
Niflumic acid	$y = (0.126702 \pm 0.004496)x + (0.01497 \pm 0.023704)$	0.9981	0.6	1.9
Urine				
Niflumic acid	$y = (0.099933 \pm 0.003064)x + (0.026986 \pm 0.079886)$	0.9972	2.6	8

* x = µg/mL of the analyte, and y = peak area of analyte.

HPLC method

Each sample was analyzed in duplicate. For each day of the experiment, the mean urine niflumic acid concentration was calculated based on the HPLC analysis of the urine samples provided for that day from the five participants.

Reagents and materials. HPLC-grade methanol and acetonitrile were supplied by Carlo Erba (Milano, Italy). Ammonium acetate of analytical grade was supplied by Riedel-de Haen (Buchs, Switzerland). A MilliQ® purification system (Millipore, Bedford, MA) was used throughout the study to provide ultrapure water.

Instrumentation. Mobile phase was delivered to the analytical column with a Shimadzu LC-10AD pump (Kyoto, Japan). Sample was injected via a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA) with a 20-µL loop. Detection was achieved at a wavelength of 288 nm and sensitivity setting of 0.002 AUFs using an SSI 500 variable UV-vis detector (SSI, State College, PA). Degassing of solvents was achieved by helium sparging prior to use.

An Inertsil ODS-3 analytical column (250 × 4.0 mm, 5 µm) purchased from MZ-Analysentechnik (Mainz, Germany) was used for the separation at ambient temperature. Mobile phase consisted of 0.05 M CH_3COONH_4/CH_3CN (40:60, v/v) and was delivered isocratically at a flow rate of 1.0 mL/min. Inlet pressure observed was 70 kg/cm^2.

Preparation of standards. A 2500 µg/mL stock solution of niflumic acid was prepared by dissolving the appropriate amount of the homogenized content of one niflumic acid capsule in 100 mL of methanol. Working standards were prepared in the range of 1.25–50 µg/mL. Solutions were stored at 4°C. Urine samples spiked at appropriate concentrations, which were used for calibration, were prepared by diluting the stock solution with drug-free urine.

Sample preparation. Urine samples were filtered with nylon microfilters, and 50 µL of the filtrate was injected onto the HPLC column.

Method validation. Calibration curves with their respective correlation coefficients, slopes, and intercepts resulted from linear regression analysis. The method was fully validated in terms of linearity, precision, and accuracy. Linearity was studied by plotting the calibration curves using drug-free urine samples spiked at concentrations in the range of 1.25–50 µg/mL. Calibration curves were constructed from the peak area of the analyte. The calculation of the slope, the intercept, and the correlation coefficient of each calibration curve was achieved through linear regression analysis. Limits of detection (LOD) were calculated from the calibration curve according to the formula LOD = 3.3 σ/S and limits of quantitation (LOQ) according to the formula LOQ = 10 σ/S, where S is the slope and σ is the standard deviation of the intercept (Table II). Precision (relative standard deviation) was evaluated at three concentrations for the urine matrices, by expressing the standard deviation of repeated measurements as a percentage of the mean value. Four replicates were used to estimate the within-day precision. Between-day precision was estimated from duplicate measurements of freshly prepared control samples during a sequence of five consecutive days. The accuracy was expressed as a percentage recovery by comparing the found concentration (concentration corresponding to the measured peak area of the analyte as calculated from the calibration curves by linear regression analysis) to the actual one (Table III).

Table III. Within-Day Repeatability, Between-Day Precision, and Accuracy for the Determination of Niflumic Acid in Urine

		Within-Day (n = 4)			Between-Day (n = 5)		
Analyte	Added (µg/mL)	Mean ± SD (µg/mL)	RSD	Recovery %	Mean ± SD (µg/mL)	RSD	Recovery %
Niflumic acid	2	1.8 ± 0.06	3.1	90.0	1.8 ± 0.06	3.5	90.0
	3	3.3 ± 0.1	4.3	110.0	2.8 ± 0.5	19.3	93.3
	5	6.1 ± 0.3	5.3	105.2	6.0 ± 0.1	2.4	120.0

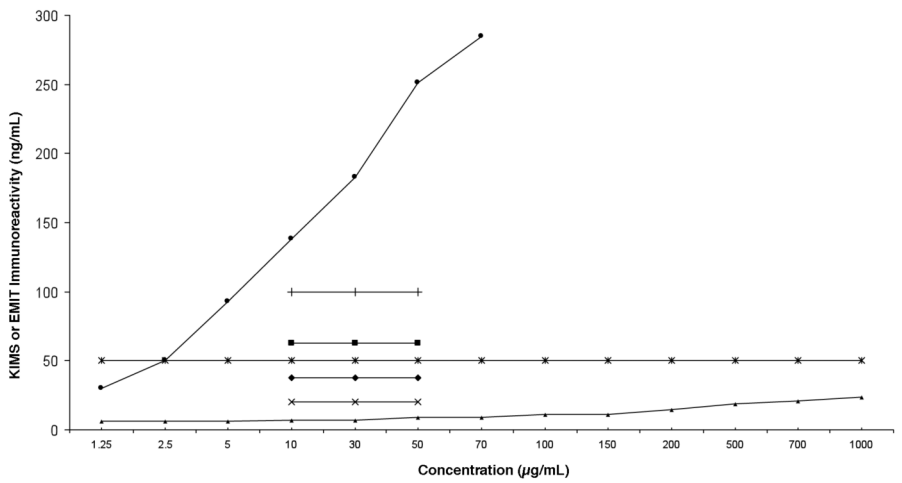

Figure 2. Immunoreactivity of niflumic acid standards when analyzed by the KIMS (♦ = low control, ■ = high control, * = cutoff calibrator, ● = niflumic acid standards) and the EMIT (× = low control, + = high control, * = cutoff calibrator, ▲ = niflumic acid standards) immunoassay.

Results

Immunoreactivity of niflumic acid standards in the KIMS and the EMIT assay is shown in Figure 2. A

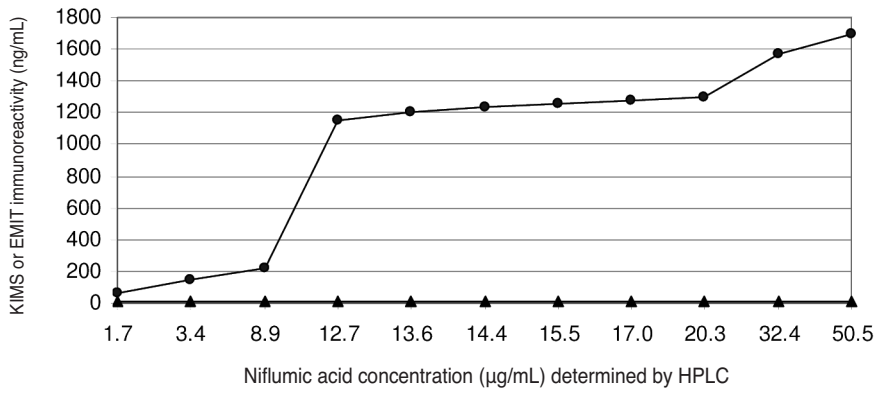

Figure 3. Immunoassay response by KIMS (●) and EMIT (▲) methodology to urine specimens in correlation to their niflumic acid concentrations as determined by HPLC.

niflumic acid concentration of approximately 2.5 µg/mL had a KIMS response equal to the 50 ng/mL cutoff (2% cross-reactivity). All standards except one gave a false-positive result when analyzed by KIMS. Cross-reactivity of niflumic acid standards in the EMIT assay (Figure 2) was very low, and all standards gave a response that was significantly lower than that corresponding not only to the 50 ng/mL cutoff calibrator, but also to the 20 ng/mL low control.

All 55 urine samples screened for cannabinoids were negative when analyzed by the EMIT assay and "presumptive" positive when analyzed by the KIMS assay (Table I). Mean niflumic acid concentration in urine for each day, as determined by HPLC (Table I), ranged from 1.7 to 50.5 µg/mL. The correlation of the immunoassay response by KIMS and EMIT methodology of urine specimens with their niflumic acid concentration, as determined by HPLC, is shown in Figure 3.

Baseline urine specimens were available for all five participants. These specimens were screened for cannabinoids both by the KIMS and the EMIT assay, and results were negative.

Discussion

Our results showed that immunoreactivity of niflumic acid standards was high when analyzed by the KIMS urine cannabinoids assay and very low when analyzed by the EMIT urine cannabinoids assay.

When analyzed by the KIMS assay, almost all standards gave a presumptive positive result, and the KIMS rate increased almost linearly with the standard concentration. It is interesting to point out that the 2% cross-reactivity we found is much lower than that reported in the package insert for the analyzer utilizing the KIMS methodology.

For all standards tested, immunoreactivity in the EMIT assay was very low, and the response was in every case much smaller than that corresponding to the 50 ng/mL cutoff calibrator.

When real urine samples were screened, the conclusions were similar. Once again, all samples collected were false positive for cannabinoids by the KIMS assay and negative (well below the low control) by the EMIT assay. It is interesting to point out that even urine samples collected days after the discontinuation of niflumic acid were presumptive positive for cannabinoids by the KIMS assay.

In view of the results, it is evident that the KIMS cannabinoids assay is more prone to cross-reactions from niflumic acid compared to the EMIT assay.

Although immunoassays are widely used in screening for drugs of abuse, their susceptibility to interfering substances, such as other classes of drugs, is a major drawback, and all presumptive positive results should be confirmed.

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