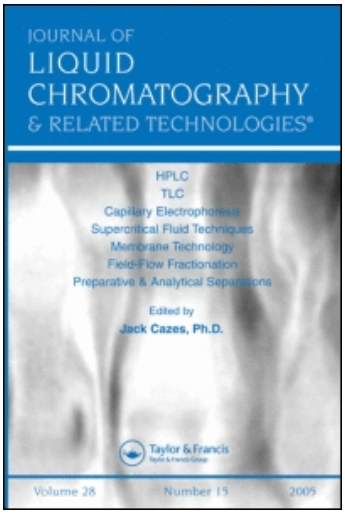

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DETERMINATION OF METFORMIN IN CULTIVATED PLANT SPECIES AND SOIL BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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□ *A liquid chromatographic-mass spectrometry (LC-MS/MS) method for the determination of metformin in cultivated plant species and soil is described. Plant samples (barley, barley-leaf, carrot, etc.) or soil were homogenized with an extraction solution of ammonium acetate/formic acid and centrifuged. The supernatant was clean-up with a solid phase extraction column, filtered, and injected into the LC/MS-MS. The limits of quantification were 30 ng/g for metformin.*

Keywords cereals, LC-MS/MS, metformin, plants, root vegetables, seed, soil

INTRODUCTION

Metformin is a biguanid anti-diabetic drug used worldwide for the treatment of type 2 diabetes mellitus, a disease characterized by defects in both insulin secretion and insulin sensitivity.[1,2] In Norway, metformin were used for the treatment of 91068 people in 2008.[3] In Germany, 416 million defined daily doses of metformin were prescribed in 2007 (more than 830 tons).[4] Metformin is absorbed relatively quickly by the intestine. About 90% of the drug is eliminated by glomerular filtration and tubular secretion with a serum half-life between 1.5 and 5 hours.[5] It is estimated that only 2% metformin is removed by wastewater-treatment.[6] Consequently, the drug will be continually introduced into the aquatic environment. In a US-study from Atlanta, metformin was detected in treated wastewater effluent/source water.[7] The use of irrigation water containing low concentrations of pharmaceuticals in agriculture may contaminate culture plants with drug residues. In Norway, the Ministry of the

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Environment has listed metformin as a substance with a potential environmental risk.[8]

The project from the Research Council of Norway no. 1848339/I10 "From plants to humans—plant accumulation and transfer of organic foreign compounds in primary food chain" will examine the possible contamination of cultivated plants with metformin.

Several analytical methods based on HPLC, LC-MS/MS are employed for the determination of metformin concentration in plasma.[9–14] However, no published methods for analyzing metformin in agriculture plant products were found. The purpose of the present study was to develop a simple, specific, and sensitive LC-MS/MS method for the determination of metformin in cultivated plants and soil.

EXPERIMENTAL

Materials and Reagents

Drug-free carrot, barley, barley leaf, and soil were used. These samples were used as control material and for spiking with metformin to conduct recovery experiments. The plant samples were stored by room temperature, but soil was stored frozen (−20°C).

All chemicals and solvents were of analytical or HPLC grade. Metformin was supplied by Sigma-Aldrich (Steinheim, Germany). Stock solution (1 mg/mL) and 10 μg/mL were prepared by dilution with water. Working standards (0.1 μg/mL) were prepared by dilution with solution A.

Solution A consisted of 0.5 M ammonium acetate-formic acid (9 + 1).

Ammonium acetate and formic acid (98–100%) were supplied from Merck (Darmstadt, Germany).

Solid phase extraction (SPE) columns Bond Elut (1 mL/25 mg) LMS, were purchased from Varian (Harbor City, CA, USA).

Spin-X centrifuge filter units (0.22 μm, nylon type) from Costar (Corning, NY, USA), were used for filtration.

Carbograph Extract-Clean Columns (500 mg) were supplied from Alltech (USA).

Charcoal activated extraction columns were packed in our laboratory with sorbent material (500 mg) supplied by Reidel-de Haën (Germany) and appropriate frits were supplied by Analytichem International (Harbor City, CA, USA).

Chromatographic Conditions

The LC-MS/MS instrumentation used for the present method consisted of a Series 200 micro pump and autosampler (Perkin Elmer, Norwalk,

TABLE 1 Mobile Phase Operating Conditions

Total Time (min)	Flow Rate (μL/min)	Solution B(%)	Solution C (%)	TE#1
0.0	800	100		Open
2.0	800	100		Open
2.1	800	27	73	Open
3.0	800	27	73	Close
3.5	800	27	73	Open
3.6	800	100		Open
4.6	800	100		Close
12.0	800	100		Open

TE#1 = events.

USA) and an API 2000 MS/MS system (Applied Biosystems, Ontario, Canada) equipped with a Turbo-Ion Spray ion source. The turbo probe vaporizer temperature of the interface was fixed at 450°C. The MS was set to collect ion data in the positive mode. Data were acquired in the multiple reaction monitoring (MRM) modes. The fragments m/z 60.1, 68.1, and 71.1 were found in MS experiments. The most abundant transitions of the protonated molecular ion m/z 130.2 to m/z 71.1 were used for screening and confirmation of the identity, while the product ions of m/z 60.1 were used for quantification.

A precolumn filter A-138 with an A-102X frits (Upchurch Scientific, USA) was connected to the guard column. The columns, Allure PFP Propyl 5 μm 150 × 4.6 mm (Restek, Bellefonte, USA, Catalog no. 9169565-700) were operated at a constant temperature of 23°C. The mobile phase consisted of a mixture of two solutions: solution B consisted of 984 mL water, 15 mL methanol, and 1 mL formic acid, and solution C was 0.1% formic acid in methanol. The mobile phase operating conditions are shown in Table 1. After separation, the LC effluent was connected to a two position micro electric valve actuator (Vici, Valco Instruments. Co. Inc. Texas, USA) programmed in mode two by our provider. Thereafter, the LC fluent was split approximately 1:4 before entering the MS interface.

Sample Pretreatment

Barley, Barley Leaf, Carrot, etc.

A volume of 1.9 mL solution A or standards for spiked samples (the corresponding volumes of standard solution were diluted to 1.9 mL with solution A) were added to 0.1 g lyophilized grind sample in a glass tube. Thereafter, 2 ml chloroform was added. The mixture was homogenized for approximately 15 sec with an Ultra-Turrax S 25N −10 G dispersing tool (Ika – Warke, Staufen, Germany) and left in an ultrasonic bath for

5 min. The sample was shaken for 3 sec and then centrifuged for 5 min at 3600 rpm. Thereafter, 0.35 mL of the water based supernatant was loaded into a conditioned LMS-SPE column.

Soil

A volume of 5 mL solution A or standards for spiked samples (the corresponding volume of standard solution was diluted to 5 mL with solution A) was added to 1 g soil. The sample was shaken vigorously by hand for 30 sec and left in an ultrasonic bath for 5 min. Thereafter, 2 mL chloroform was added. The sample was shaken for 10 sec and then centrifuged for 5 min at 3600 rpm. Then, 0.35 mL supernatant of the water based supernatant was loaded into a conditioned LMS-SPE column.

Clean-Up on SPE-Column

The column was conditioned with 1 mL methanol, followed by 2×1 mL water and suctioned to dryness (−10 in. Hg.) for 5 sec. The water extract (0.35 mL) from cultivated plants or soil was loaded into the column and slowly suctioned through with vacuum circa −1 in. Hg. Thereafter, the column was suctioned to dryness (−5 in. Hg.) for 10 sec. The entire loaded water sample that passed through the column was collected, blended, and filtered through a Spin-X centrifuge filter by centrifugation for 2 min (10 min for barley) at 10000 rpm ($5600 \times g$). Aliquots of 20 µL were injected into the LC-MS/MS system for the determination of metformin.

Removal Metformin in Water

A volume of 1 mL metformin water based standard (5 µg/mL) was loaded into a carbograph extract-clean column (500 mg) or a self packed charcoal activated column. The sample was pressed through the column by a syringe. The entire water sample that passed through the column was collected (Fraction 1). A new 1 mL, but now with pure water, was passed through the column and collected separately (Fraction 2). This procedure was repeated 6 times, making 8 different fractions. Aliquots of 20 µL were injected into the LC-MS/MS for the determination of metformin for each fraction.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for metformin was determined by spiking drug-free barley, barley leaf, carrot, and soil samples with standard solutions to yield 0, 25, 30, 50, 100, 200, 400, 500, 800, and 1000 ng/g.

Duplicate samples were used. The recovery was determined by comparing the analyses of spiked samples with those of standard solutions of metformin. The standards were prepared by diluting the standard with drug-free extract from the different plant species or soil. The linearity of the standard curves for metformin was calculated using peak area measurements.

For the determination of recovery rates of metformin, the corresponding doses of standard solutions were diluted to 2 mL for cultivated plant species and to 6 mL for soil.

RESULTS AND DISCUSSION

The standard curves were linear in the investigated areas from 30 to 1000 ng/g for barley, barley leaf, carrot, and soil. The linear coefficients for metformin in barley, barley leaf, carrot, and soils was 0.9997 and the recovery and repeatability values for metformin are shown in Table 2. The recovery was calculated directly, without correction for an internal standard. The matrix effect from the different plants species were evaluated at two levels (100 and 800 ng/mL), comparing standards diluted in water with standard solutions diluted in drug-free extract from barley, barley leaf, carrot, and soil. Between the different matrixes significant matrix effects were observed. When analyzing specific matrixes, it is important to use the corresponding standard curve from the specific matrix. Chromatograms obtained from drug-free samples and from real samples from cultivated, contaminated plants with metformin are shown in Figures 1–3.

The SPE – LMS columns were used to remove undesirable endogenous compounds. The use of SPE – LMS columns resulted in a more robust method.

TABLE 2 Recovery and Repeatability for Metformin from Spiked Samples of Barley, Barley Leaf, Carrot, and Soil

No. of Sample	Amount of Drug (ng/g)	Recovery (%) Mean	Metformin S.D.
Barley			
6	100	98	0.3
6	800	99	0.3
Barley leaf			
6	100	66	1.0
6	800	66	0.2
Carrot			
6	100	94	1.5
6	800	95	0.2
Soil			
6	100	66	0.6
6	800	73	0.1

S.D. = Standard deviation.

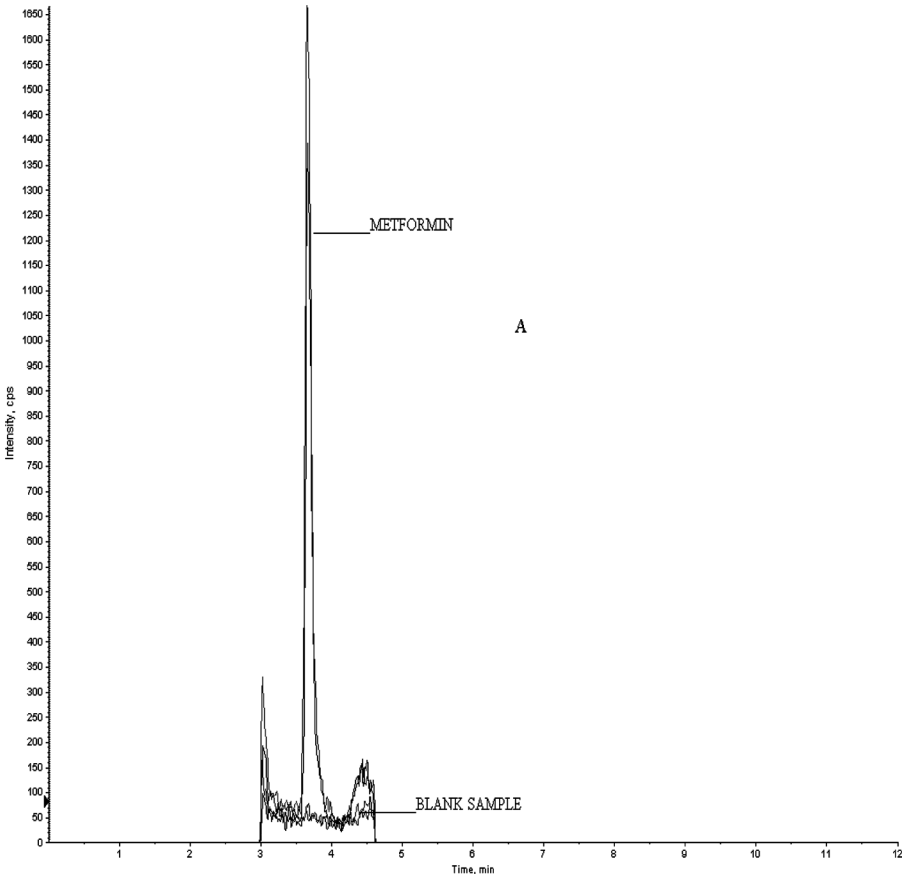

FIGURE 1 LC-MS/MS chromatograms (A) of a blank extracts and from a real sample of barley-leaf containing 1.69 μg/g metformin.

The limits of detection for metformin were calculated as three times the peak-to peak baseline noise (S/N = 3) from drug-free samples. They were 20 ng/g for metformin for barley, barley leaf, carrot, and soil. The limit of quantifications was 30 ng/g for the four matrixes.

The extraction of metformin from cultivated plants and soil involved acid to release the bound compound. Chloroform was added to ease the absolutely necessary homogenizing step with an Ultra-Turrax. In this way, the contact surface between the extraction solution and the matrix sample was increased. Also, chloroform tie plant dye and eases the separation of water from the solid phase.

The use of a two position micro electric valve actuator avoided use of unnecessary mobile phase and, thereby, eliminating possible contamination from sample extracts streaming into the MS. It is essential that

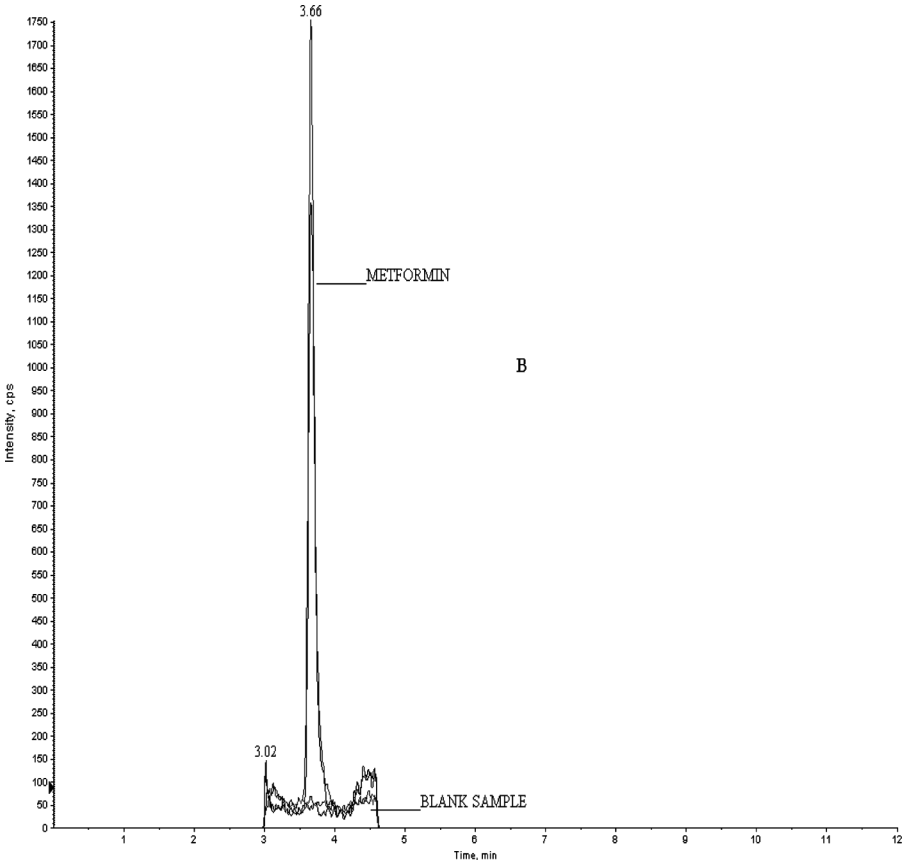

FIGURE 2 LC-MS/MS chromatograms (B) of a blank extracts and from a real sample of barley-seed containing 0.731 μg/g metformin.

the micro electric valve actuator is programmed in mode two. The actuator is guided from data software under LC pump (events). When the event is open, the mobile phase flows to waste. When the event is closed, the mobile phase flows to the MS until a new close event is given; hereafter, the mobile phase flows to waste. However, the use of a micro electric valve actuator is not an absolute requirement to carry out the described method for metformin.

Treatment of drinking water to reduce the concentrations of chemical pollutants includes a variety of processes.[15] Activated carbon, either powdered or granular, has a significant affinity for organic compounds. In humans, metformin intoxication with the development of severe acidosis is reported. Activated charcoal is recommended as one of several treatment regimes.[16,17] We studied the removal of metformin from water by activated charcoal in a small laboratory experiment. Two columns were

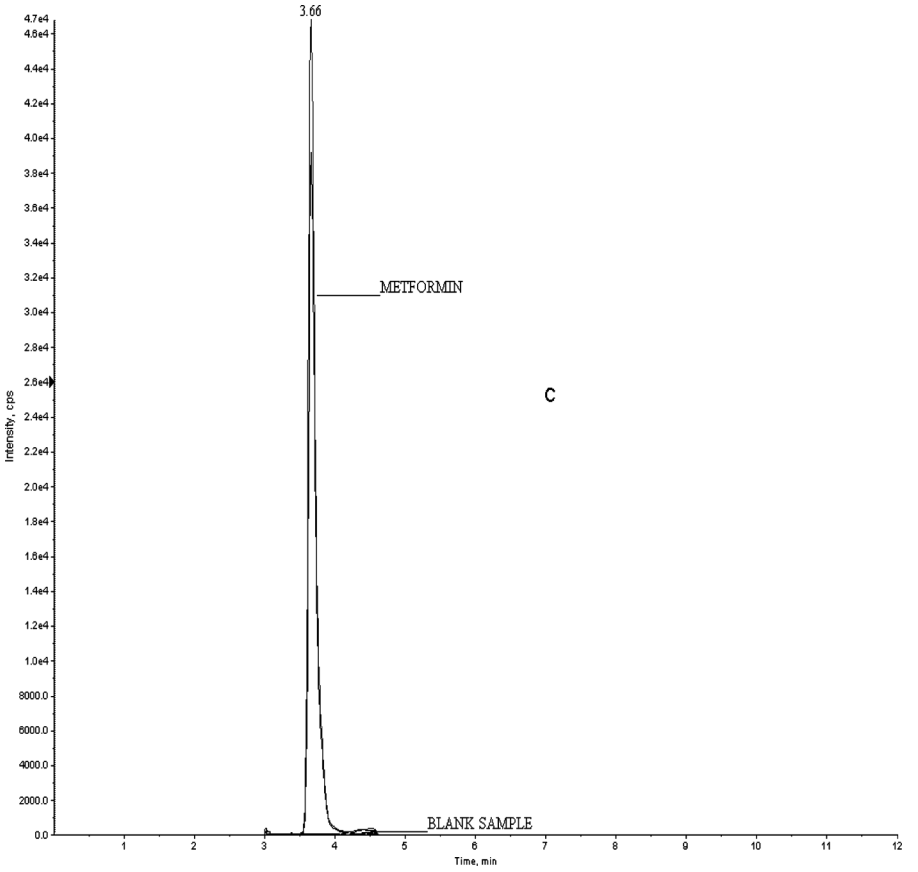

FIGURE 3 LC-MS/MS chromatograms (C) of a blank extracts and from a real sample of carrot containing 36 μg/g metformin.

used; carbograph extract-clean 500 mg column and a self-packed column with 500 mg pure, activated granular charcoal. The results indicated that activated charcoal had limited effect on removal of metformin from water (Figure 4). The two columns tested showed similar results.

Metformin is a very polar hydrophilic compound and, therefore, difficult to retain on analytical columns. Polar columns used with highly organic mobile phases provide a normal-phase separation mechanism, leading to sufficient retention of polar substance for determination of metformin in plasma samples.[14,18] In these studies, retention times of 0.6 and 1.6 min were used. In the present method, longer retention time are need for analyzing plant species and soil. Pentafluorophenyl HPLC phases show greater retention for compounds that have electrophilic properties, like protonated amino groups in basic compounds.[19] A propyl spacer between the functional group and the silica surface, a penta-fluorophenyl propyl phase,

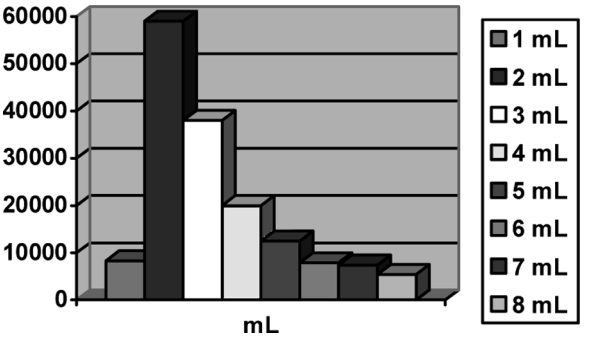

FIGURE 4 Release of metformin from activated carbon columns in 7 samples of water after application of 5 µg metformin (1 mL).

further increases retention. Consequently, when an acidic mobile phase is used to induce protonation of the analytes amino groups, the Allure PFP Propyl phase makes possible a simple reversed phase HPLC analysis.

The LC-MS/MS method presented here was selective and robust. No co-extracts with interfering peaks were found. The time needed for sample preparation was short.

CONCLUSIONS

The applications presented here provide good evidence that LC-MS/MS can offer a number of significant advantages for the detection and quantification of metformin in plant species and soil. The advantage of the LC-MS technique lies in the combination of the separation capabilities of HPLC and the power of MS as an identification and confirmation tool with good sensitivity, selectivity, and quantitative capability. The LC-MS/MS methods generally require only a simple clean-up or only a dilution procedure and no derivatization. The validation data showed that the method performance is good and can be used for routine analysis.

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