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Determination of phytoestrogens in bovine plasma using liquid chromatography and tandem mass spectrometry

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ABSTRACT

Phytoestrogens have received an increasing interest due to their potential protective effect against a number of human diseases. A method based on liquid-chromatography and negative electrospray tandem mass spectrometry was developed for assaying phytoestrogens in blood plasma. The detection limit was 0.1 to 3.1 nM for various isoflavones, lignans and mycotoxines. In a dairy cow fed whiteclover grass the arterial plasma concentrations of the conjugated forms of enterodiol, enterolactone, daidzein, and equol were 2, 440, 93 and 115 nM, respectively. The concentrations of the non-conjugated forms were 0.4, 16, 0.8 and 1.1 nM, respectively. Formononetin and genistein were detected only in the conjugated forms (1.6 and 22 nM). There were positive net portal fluxes of all phytoestrogens detected. The liver extracted equol, but released daidzein in the non-conjugated form. These preliminary data indicate important differences in liver metabolism of various phytoestrogens in dairy cows.

KEY WORDS: phytoestrogens, HPLC, bovine plasma, mass spectrometry

INTRODUCTION

In the recent years, phytoestrogens (isoflavones, coumestans, lignans and mycotoxines) have received an increasing interest. The interest is focused on their potential protective effect against a number of complex diseases common in Western societies, like certain cancer forms and their possibly beneficial effect on osteoporosis and menopausal syndrome. Concerns have, however, been raised that consumption of phytoestrogens by infants and young children may be undesirable. Dairy cows fed leguminous plants like clover and soya may excrete phytoestrogens

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in milk, which then may be a source of phytoestrogens in the human diet. In milk samples the concentration range has been demonstrated to be 1-3000 nM for equol and 0.1-30 nM for biocanin A, daidzein, genistein, and formononetin. The lignans were in the range of 0-40 μM- (Hansen-Møller et al., 2004).

The aim of the present study was to develop a simple and sensitive method for determination of phytoestrogens in bovine plasma without time consuming solid phase extraction or solvent extraction. The isoflavones of interest include: biochanin A, daidzein, equol, formononetin, genistein and prunetin while the lignans include enterolactone and enterodiol and the coumestan: coumesterol. The mycotoxines include zeralenone as well as two of the metabolites of zeralenone: α-zeralenol and β-zeralenol.

MATERIAL AND METHODS

Biochanin A, daidzein, equol, formononetin, genistein, prunetin enterolactone, enterodiol, coumesterol, zeralenone, α-zeralenol, β-zeralenol and β-glucuronidase/ arylsulphatase Type HP-2 *Helix pomatia* were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ammonium hydroxide, and acetic acid were obtained from Merck (Darmstadt, Germany).

A set of blood samples were collected from a dairy cow fitted with permanent indwelling catheters in the mesenteric artery, hepatic portal vein, and hepatic vein. The cow was fed as described by Røjen et al. (companion paper this issue). Five-hundred μl of blood plasma was combined with an equal volume of 0.2 M ammonium acetate buffer pH 5.2 and 10 μl β-glucuronidase/ arylsulphatase. Conjugates of the phytoestrogens were cleaved by incubating at 40°C for 3 h. Proteins were partly precipitated by adding 20 μl methanol followed by a centrifugation at 20.000 × *g*. Fifty microlitre samples were injected into a column-switching system on a HPLC (Agilent, Waldberon, Germany) equipped with a well plate sampler, temperature controlled column compartment with column-switching valve, and a high-pressure binary gradient pump. The flow rate was 0.4 ml/min. The phytoestrogens were separated on a Zorbax XDB column (3.5 μm, 3 × 150 mm, Agilent) using a gradient between solvent A: 0.5% acetic acid and B: methanol going from 50% B to 74% B in 14 min. A third mobile phase C consisting of ammonium acetate pH 5.2, methanol and H_2O (10:200:790 v/v) was pumped through the column switching system, at a flow rate of 0.6 ml/min, using an LKB 2248 pump (Pharmacia, Uppsala, Sweden). The column switching system was constructed using two 6-port Rheodyne valves (Cotati, CA, USA) and an Alexa 30 μ (Ansys, Torrance, CA, USA) trapping column (50 mm × 3 mm) as described by Hansen-Møller et al. (2004). The precolumn system was used for desalting of the samples as well as removal of peptides from the samples prior to separation in the HPLC system.

A Quattro LC from Micromass (Manchester, UK) was used. The settings for the negative electrospray were: Desolvation gas (N_2) 550 l/h at a temperature of 350°C, capillary voltage -3.0 kV and source temperature 120°C. Argon was used as collision gas in the MRM method (multiple reaction monitoring; 2.5 mTorr). The cone voltage as well as the collision energy was set individually for each of the phytoestrogens (Table 1). The analytes were quantified by means of calibration curves formed from known concentrations of the analytes ranging from 1 to 100 nM.

Table 1. Conditions for the MRM method (multiple reaction monitoring) used by the tandem mass spectrometry detection of phytoestrogens in bovine plasma

	Precursor ion	Daughter ion	Cone V	Collision eV	LOD[a] nM	Rt[b] min
Enterodiol	301.0	253.1	35	22	0.3	6.70
Daidzein	252.8	91.1	45	40	0.7	6.99
Enterolacton	297.0	107.0	35	27	1.0	7.35
Equol	240.9	119.0	25	32	1.0	7.70
Genistein	268.8	133.0	40	33	0.7	8.35
Formononetin	266.9	252.0	35	22	0.1	10.61
β-Zeralenol	319.0	130.0	29	35	1.0	11.40
Prunetin	282.8	268.0	30	20	0.5	11.93
Coumesterol	266.9	91.1	40	38	3.1	11.94
Biochanin A	282.9	211.0	28	38	0.1	12.64
α-Zeralenol	319.0	130.0	35	31	1.0	13.10
Zeralenone	317.2	131.0	36	30	1.4	13.63

[a] limit of detection corresponds to a signal to noise ratio of 3
[b] retention time of the corresponding compound in the HPLC system

RESULTS AND DISCUSSION

By the developed method it was possible to detect both conjugated and non-conjugated forms of enterodiol, enterolactone, daidzein, genistein, equol, and formononetin in bovine blood plasma except for formononetin and genistein, which were only detected in the conjugated form (Table 2). The method also worked for coumersterol, prunetin, biocanin A, chrysin, α-zeralenol, β-zeralenol, and zeralenone, but these compounds could not be detected in the bovine blood plasma. The sensitivity of the method is comparable to those reported by Grace et al. (2003) for human plasma.

The conjugated forms of the phytoestrogens were found in six- to hundredfold higher concentration than the non-conjugated forms. Higher portal than arterial concentrations indicate that both conjugated and non-conjugated phytoestrogens were absorbed from the gut. The liver did not efficiently extract any of the detected compounds. For daidzein the liver even seemed to have a net release of the non-conjugated form and a net uptake of the conjugated form. The liver

appeared to have a net uptake of the other non-conjugated phytoestrogens and will therefore decrease the exposure of the peripheral tissues of some phytoestrogens but increase the availability of daidzein. The biological implications of these differences are unknown.

Table 2. Concentrations (nMl) of phytoestrogens in arterial, portal, and hepatic blood plasma from a dairy cow fed a whiteclovergrass based ration

Compound	Blood plasma sample		
	artery	portal	hepatic
Total concentration (conjugated + non-conjugated) of phytoestrogens			
enterodiol	2.3	3.7	3.4
enterolactone	440	485	485
daidzein	93	184	158
equol	115	150	148
genistein	22	42	35
formononetin	1.6	2.5	2.2
Concentration of non-conjugated phytoestrogens			
enterodiol	0.4[b]	0.6[b]	0.5[b]
enterolactone	16	19	17
daidzein	1.1[b]	1.9[b]	3.2
equol	0.8[b]	9.7	3.6
genistein	nd[a]	nd	nd
formononetin	nd	nd	nd

[a]nd - concentration below the limit of detection (LOD) see Table 1

[b] concentration less than the limit of quantification (signal to noise ratio < 8)

CONCLUSIONS

A sensitive method for determination of phytoestrogens in bovine plasma has been established using LC-MS/MS. Samples from a single cow indicated that there might be important differences in the metabolism of different phytoestrogens by the liver.

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