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Validated method for the determination of six metabolites derived from artichoke leaf extract in human plasma by high-performance liquid chromatography–coulometric-array detection

Sabine M. Wittemer[a], Markus Veit[b,*]

[a] *Lichtwer Pharma AG, Wallenroder Strasse 8–10, 13485 Berlin, Germany*
[b] *LAT GmbH, Am Haag 4, 82166 Gräfelfingen, Germany*

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Abstract

A validated method was developed for the simultaneous determination of the hydroxycinnamates caffeic (CA), dihydrocaffeic (DHCA), ferulic (FA), dihydroferulic (DHFA), and isoferulic acid (IFA) and the flavonoid luteolin (LUT) in human plasma as metabolites derived from artichoke leaf extract. The method involves sample preparation followed by separation using high-performance liquid chromatography on reversed-phase material with a polar endcapping (Aqua-C_{18}, 250×4.6 mm). Selectivity and sensitivity towards the target compounds were achieved by electrochemical array detection (CoulArray). Calibration curves were constructed in the ranges 2.1–51.7 ng mL^{-1} (CA), 2.0–76.7 ng mL^{-1} (DHCA), 2.2–53.7 ng mL^{-1} (FA), 2.1–79.2 ng mL^{-1} (DHFA), 1.1–52.6 ng mL^{-1} (IFA) and 2.1–258.6 ng mL^{-1} (LUT). Linearity could be shown for all target compounds over the entire calibration range. Values for within-day and between-day precision and accuracy were in accordance with the international guidelines for validation of bioanalytical methods. It is concluded that this newly developed method is appropriate for analysing samples from bioavailability and pharmacokinetic studies after oral administration of artichoke leaf extract.

Keywords: Validation; Artichoke; Hydroxycinnamates

1. Introduction

Extracts from artichoke leaves are traditionally used in the treatment of dyspeptic and hepatic disorders. Efficacy has been demonstrated in several clinical studies [1,2]. Due to increased bile production into the duodenum of healthy volunteers an increase in choleresis was described by Kirchhoff et al. [3]. The symptoms of dyspetic disorder and irritable bowel syndrome (IBS) overlap, and a recent study has also shown a reduction of IBS symptoms after treatment with artichoke leaf extract [4]. Among these choleretic effects, artichoke leaf extract has also produced a decrease of cholesterol and LDL-cholesterol serum levels in patients with hyperlipoproteinemia [5]. Thus treatment with artichoke leaf extract is believed to reduce the risk of coronary heart disease and artherosclerosis. Further effects, such as inhibition of hepatocellular cholesterol biosynthesis [6,7], stimulation of biliary secretion [8]

*Corresponding author. Tel.: +49-89-8589-67120; fax: +49-89-8589-67111.
E-mail address: veit@lat-gmbh.de (M. Veit).

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and antioxidant activities [9], have been observed in different in vitro assays. Luteolin and luteolin-7-*O*-glucoside as well as 1,5-dicaffeoylquinic acid and several other polyphenolic extract constituents such as caffeic acid and chlorogenic acid are considered to be active antoxidants [10,11]. In contrast, inhibition of cholesterol biosynthesis and bilinary secretion was observed only for luteolin and to a lesser extent for luteolin-7-*O*-glucoside, while 1,5-dicaffeoylquinic acid and chlorogenic acid were almost ineffective [6,8]. However, in vivo, not only the genuine extract constituents, but also metabolites thereof, may contribute to efficacy. Therefore, evaluation of the systemic availability of potential bioactive plant constituents is a major prerequisite for the interpretation of in vitro pharmacological testing.

A recent in vivo study in humans evaluated ferulic acid, dihydroferulic acid, isoferulic acid and vanilic acid derived from caffeoylquinic acids as the main urinary metabolites after oral administration of artichoke leaf extract. Except for dihydroferulic acid, all of them have been found mostly conjugated to sulfuric or glucuronic acid [12]. Previous studies have demonstrated that, after ingestion of chlorogenic acid in rats, caffeic acid and ferulic acid, but not intact chlorogenic acid, were found in plasma and urine [13–15]. The bioavailability and metabolism of luteolin-7-*O*-glucoside have mainly been studied in animals. Deglucosylation to luteolin and glucuronidation during transport across the gut were observed [16].

A reliable and sensitive method for the determination of caffeic acid, dihydrocaffeic acid, ferulic acid, dihydroferulic acid, isoferulic acid and luteolin in human plasma has been developed. Thus method development included most of the metabolites reported for artichoke leaf extract constituents from humans in the literature. Preliminary investigations showed that plasma concentrations of all target compounds after oral administration of therapeutic doses of artichoke leaf extract were in the lower ng mL^{-1} range. Therefore, a very sensitive bioanalytical method was required. As HPLC–photodiode array detection (PDA) would not provide sufficient sensitivity [17–19], HPLC with electrochemical detection was chosen for analysis of the target compounds in human plasma. Electrochemical detection offers high sensitivity for polyphenols [20] and the capability of selective detection using a multi-channel array detector [21]. The multi-channel array detector consisted of 12 electrodes in series set incrementally to different potentials. A chromatogram was generated at each potential. A plot of the 12 chromatograms provided, for each compound, its specific hydrodynamic voltammogram, which was used for peak identification. For separation of the target compounds from the plasma matrix, a C_{18} phase with polar endcapping providing excellent peak shape for hydrophilic compounds with an appropriate gradient.

Instead of solid-phase extraction or liquid–liquid extraction procedures often used in bioanalytics, plasma sample preparation was performed following the method of Graefe et al. [22]. Samples were prepared by simple protein precipitation, evaporation and a redissolving step. The method was slightly modified in order to extract and detect hydroxycinnamates and flavonoids simultaneously.

The developed method was validated in accordance with international guidelines for the validation of bioanalytical methods [23], and was used for the determination of metabolites in a clinical study of the bioavailability and pharmacokinetics of artichoke leaf extract after oral administration. Only example data are shown and the study will be published in its entirety elsewhere.

2. Experimental

2.1. Chemicals and human plasma matrix

Caffeic acid (≈97%), luteolin (90%), acetone (≥99.5%), methanol (gradient grade), L(+)-ascorbic acid (≥99.5% p.a.), orthophosphoric acid (85% p.a.) and trifluoroacetic acid (≥99.9%) were obtained from Roth (Karlsruhe, Germany). Dihydrocaffeic acid (purum ≥98.0%), ferulic acid (purum ≥98.0%) and acetic acid (99.8% p.a.) were obtained from Fluka (Buchs, Switzerland). Dihydroferulic acid (98.0%) was purchased from Lancaster Synthesis (Newgate, UK) and isoferulic acid (99.3%) from Apin Chemicals (Milton Park, UK). Isoferulic acid was certified as a natural product reference substance (NPRS) at the Zentralinstitut Arzneimittelforschung (Sinzig, Germany). Sodium dihydrogenphosphate

(99%) and β-glucuronidase (EC 3.2.1.31) were purchased from Sigma–Aldrich (Deisenhofen, Germany). Blank plasma was obtained from healthy volunteers. All volunteers gave written informed consent to participate in the study.

2.2. HPLC–CoulArray system

The HPLC equipment was supplied by Environmental Sciences (ESA, Chelmsford, MA, USA) and consisted of an autosampler Model 542 (set to a temperature of 15 °C, injection volume 50 μL), a solvent delivery system Model 582, a column thermostat type 880 (set to a temperature of 25 °C), and a coulometric array detector CoulArray. The 12 cells of the electrochemical detector were set from 75 to 900 mV in increments of 75 mV. Determination of the target compounds was carried out at 150 mV (DHCA, LUT), 225 mV (CA), 375 mV (DHFA, FA) and 450 mV (IFA). For separation, a column (250× 4.6 mm I.D., 5 μm) packed with C_{18}-Aqua (octadecylsilane with polar endcapping) from Phenomenex (Aschaffenburg, Germany) was used.

The solvents (all of gradient grade) were a 0.02 *M* aqueous solution of sodium dihydrogenphosphate adjusted to pH 2 with orthophosphoric acid (A) and methanol–0.1 *M* aqueous solution of sodium dihydrogenphosphate (4:1, v/v) containing 0.25% orthophosphoric acid (B). The flow-rate was set to 0.8 mL min^{-1}. For separation of the target compounds from the plasma matrix the solvent gradient began at 28% B, increasing to 33% B after 5 min, held until 30.5 min, increasing to 45% B at 50 min, held until 57.5 min and increasing to 100% B at 87.5 min. The gradient was followed by 10 min column flushing and post-run equilibration, respectively. The chromatograms were recorded using ESA CoulArray Win software version 1.02.

2.3. Sample preparation

Fifty microliters of water (50 μL stock solution of target compounds for validation) and 10 μL ascorbic acid 0.5% (m/v) were added to 500 μL human plasma, the pH was adjusted to 5.0 with 0.58 *M* acetic acid and 30 μL of β-glucuronidase solution were added. The mixture was incubated at 37 °C for 1 h. Precipitation of proteins was carried out by adding 600 μL acetone. After vortexing for 2 min the mixture was centrifuged for 10 min at 7830 *g*. The supernatant was transferred to a cup containing 10 μL ascorbic acid 0.5% (m/v) and 20 μL 1 *M* trifluoroacetic acid and evaporated in a vacuum centrifuge until dry. The residue was redissolved in 150 μL methanol–water (1:1, v/v) and centrifuged for 10 min at 7830 *g*. The supernatant was used for HPLC–CoulArray analysis.

As blank plasma was not completely free from the target compounds after β-glucuronidase treatment, method validation was performed without adding the enzyme, but including all other steps.

2.4. Validation parameters and procedures

Validation was performed in accordance with the ''FDA Draft Guidance for Industry No. 2578 (Bioanalytical Methods Validation for Human Studies, 1998)'' regarding selectivity, limit of quantification (LOQ), calibration curve, linearity, precision, accuracy, recovery and autosampler stability. For validation purposes a homogenous plasma matrix including blank plasma from nine volunteers was mixed and spiked with a standard solution containing all target compounds. The standard solution was prepared by diluting a methanolic stock solution further with water.

3. Results

3.1. Sample preparation

Sample preparation followed the method of Graefe et al. [22]. As this method was developed for the determination of the flavonol quercetin in human plasma, it was not possible to detect hydroxycinnamates in an appropriate way. For these compounds, misshaped peaks were obtained in the chromatograms. Most likely, the sample solution was not sufficiently acidic and hydroxycinnamates were present as protonated and nonprotonated species. However, the peak shape could be improved by adding 20 μL of 1 *M* trifluoroacetic acid to each sample before evaporation. To increase the solubility of the hydrophilic hydroxycinnamates the final solvent used to redissolve the residue was changed from water–

dimethylformamide (1:2, v/v) to water–methanol (1:1, v/v).

3.2. Selectivity

Homogenous blank plasma from nine volunteers was prepared with and without β-glucuronidase treatment and compared with a standard solution of DHCA, CA, DHFA, FA, IFA and LUT. Blank plasma treated with β-glucuronidase showed minor peaks separated from proximate peaks at the retention times of all target compounds except for isoferulic acid. As the hydrodynamic voltammograms of the minor peaks and the target compounds were identical, it was assumed that the blank plasma was not completely free of DHCA, CA, DHFA, FA and LUT, although it was obtained after 2 days on a plant material-free diet. However, chromatograms from blank plasma prepared without β-glucuronidase compared with chromatograms derived from blank plasma spiked with CA, DHCA, FA, DHFA, IFA and LUT at their respective LOQs showed no further interferences (Fig. 1).

3.3. Calibration curve and linearity

Calibration curves were repeatedly constructed with a minimum of six concentration points over the ranges 2.1–51.7 ng mL^{-1} (CA), 2.0–76.7 ng mL^{-1} (DHCA), 2.2–53.7 ng mL^{-1} (FA), 2.1–79.2 ng mL^{-1} (DHFA), 1.1–52.6 ng mL^{-1} (IFA) and 2.1–258.6 ng mL^{-1} (LUT) by spiking known quantities of target compounds into blank plasma. Samples were prepared according to the sample preparation procedure described previously. Peak areas of electrochemical signals were plotted against concentration for each target compound. Linearity was found over the entire calibration range. Correlation coefficients R^2 ($n=3$) were in the range 0.9993–0.9998 (CA), 0.9923–0.9998 (DHCA), 0.9978–0.9993 (FA), 0.9856–0.9992 (DHFA), 0.9981–0.9994 (IFA) and 0.9959–0.9995 (LUT) (Table 1).

3.4. Limit of quantification (LOQ)

The limit of quantification was defined as the lowest concentration on the standard curve that could be measured with acceptable precision and accuracy. Thus the LOQs were 2.1 ng mL^{-1} (CA), 2.0 ng mL^{-1} (DHCA), 2.2 ng mL^{-1} (FA), 2.1 ng mL^{-1} (DHFA), 1.1 ng mL^{-1} (IFA) and 2.1 ng mL^{-1} (LUT). Precision and accuracy at the LOQ met, for all target compounds, the requirements (relative standard deviation, RSD and accuracy, RE $\leq$20%) generally accepted in bioanalytical analysis [23], as shown in Tables 2 and 3.

3.5. Precision and accuracy

Precision was determined at three different concentrations covering the calibration range for each target compound. RSDs of the peak areas from five measurements were calculated for within-day precision. The determination was repeated on a second day for the between-day precision. For accuracy estimation, five replicate samples were run at three different concentrations. It was calculated as the percentage of the RSD of the actual value from the true value. All data obtained for precision and accuracy presented in Tables 2 and 3 met the criteria required for bioanalytical method validation proposed by the draft document of the FDA [23].

3.6. Recovery

Recovery of all target compounds was determined at three concentrations covering the calibration range. Peak areas obtained from spiked blank plasma samples with known concentrations were compared with peak areas obtained from solutions injected directly. Determination of all target compounds prepared using the same sample preparation method led to recoveries of at least 62.2% (Table 4).

3.7. Autosampler stability

Plasma samples for autosampler stability tests were prepared in duplicate at a concentration of 25 ng mL^{-1} for each target compound and stored in the autosampler tray at 15 °C. Stability was monitored after 24, 48 and 72 h. Due to the ascorbic acid content, all target compounds were stable for at least 72 h in human plasma matrix. Changes from the initial value after this time amounted to 0.8% (CA), 3.4% (DHCA), 5.5% (FA), 3.2% (DHFA), 4.4% (IFA) and 0.3% (LUT).

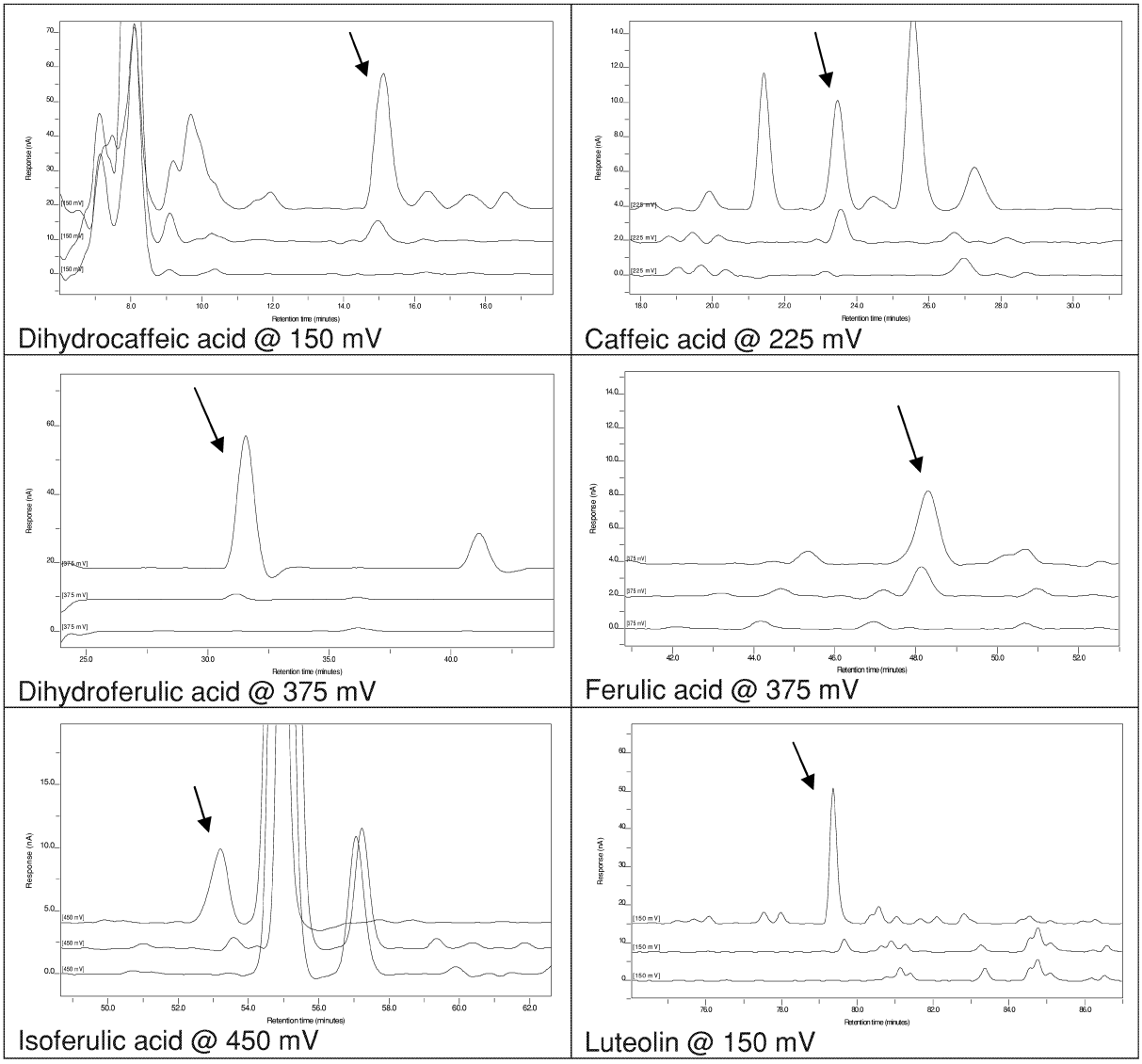

Fig. 1. Details of the chromatograms of blank plasma, blank plasma spiked with target compounds and plasma after application of artichoke leaf extract.

3.8. Example results

The presented method was used as an analytical tool for the determination of plasma metabolites in a clinical study of the bioavailability and pharmacokinetics of artichoke leaf extract components after oral administration. Data from this study will be published in detail elsewhere. Fig. 2 shows exemplary concentration versus time plots of all target compounds for one volunteer after ingestion of artichoke leaf extract corresponding to 153.8 mg caffeic acid and 35.3 mg luteolin.

4. Discussion

A reliable and sensitive HPLC method for the determination, in human plasma, of six metabolites

Table 1
Calibration curves for target compounds in human plasma ($n=3$)

	Calibration range (ng mL^{-1})	Slope (mean±SD)	Intercept (mean±SD)	R^2 (mean)
CA	2.1–51.7	28.7±2.8	5.9±2.2	0.9995
DHCA	2.0–76.7	27.5±3.3	85.3±8.6	0.9971
FA	2.2–53.7	27.0±4.1	9.3±4.6	0.9987
DHFA	2.1–79.2	32.7±1.9	0.4±1.2	0.9940
IFA	1.1–52.6	30.1±1.2	16.4±1.1	0.9988
LUT	2.1–258.6	10.5±0.6	5.4±2.4	0.9980

Table 2
Data for within-day precision and accuracy ($n=5$)

	True value (ng mL^{-1})	Day	Mean (ng mL^{-1})	RSD (%)	RE (%)
CA	2.1	1	1.7	18.4	−17.2
		2	1.7	8.6	−15.9
	15.5	1	15.3	2.9	−1.1
		2	15.4	1.3	−0.7
	51.7	1	52.4	10.4	1.5
		2	50.8	6.8	−1.7
DHCA	2.0	1	2.4	16.6	18.1
		2	2.4	16.2	15.6
	25.6	1	22.3	2.7	−12.6
		2	22.6	3.7	−11.5
	76.7	1	79.0	3.6	3.1
		2	80.7	3.1	5.3
FA	2.2	1	1.9	14.2	−13.6
		2	1.9	5.1	−13.6
	16.1	1	17.6	6.6	9.1
		2	17.5	5.0	8.9
	53.7	1	48.8	7.8	−9.1
		2	52.8	6.1	−1.7
DHFA	2.1	1	2.1	9.8	1.0
		2	2.4	16.9	11.5
	26.4	1	23.8	2.4	−10.0
		2	24.0	5.2	−9.2
	78.8	1	73.4	6.2	−6.8
		2	75.4	11.0	−4.4
IFA	1.1	1	1.0	18.1	−6.8
		2	0.9	11.7	−10.1
	15.8	1	16.9	6.7	7.0
		2	16.5	4.8	4.6
	52.6	1	52.8	9.9	0.4
		2	48.1	6.5	−8.6
LUT	2.1	1	2.0	4.6	−1.6
		2	2.1	4.7	−0.1
	103.5	1	105.0	10.8	1.5
		2	100.5	4.6	−2.9
	258.6	1	262.4	4.8	1.4
		2	263.1	5.7	1.7

Table 3
Data for between-run precision and accuracy

	True value (ng mL^{-1})	Mean (ng mL^{-1})	RSD (%)	RE (%)
CA	2.1	1.7	1.1	−16.5
	15.5	15.4	0.3	−0.9
	51.7	51.6	2.3	−0.1
DHCA	2.0	2.4	1.5	16.8
	25.6	22.5	0.9	−12.0
	76.7	79.9	1.5	4.2
FA	2.2	1.9	0.0	−13.6
	16.1	17.6	0.1	9.0
	53.7	50.8	5.6	−5.4
DHFA	2.1	2.2	7.0	6.2
	26.4	23.9	0.7	−9.6
	78.8	74.4	1.9	−5.6
IFA	1.1	1.0	2.6	−8.5
	15.8	16.7	1.7	5.8
	52.6	50.4	6.7	−4.1
LUT	2.1	2.1	1.1	−0.8
	103.5	102.7	3.1	−0.7
	258.6	262.7	0.2	1.6

Table 4
Recoveries and relative standard deviations (RSDs) for target compounds in human plasma ($n=5$)

	Conc. (ng mL^{-1})	Recovery (%)	RSD (%)
CA	2.1	80.7±2.7	3.3
	15.5	80.8±1.0	1.3
	51.7	85.4±8.8	10.4
DHCA	2.0	86.7±5.6	6.5
	25.6	80.6±2.6	3.2
	76.7	85.6±2.5	2.9
FA	2.2	62.2±5.1	8.2
	16.1	64.5±3.1	4.9
	53.7	66.2±4.1	6.2
DHFA	2.1	82.6±6.0	7.2
	26.4	70.3±2.0	2.8
	78.8	72.6±4.5	6.2
IFA	1.1	73.3±8.7	11.9
	15.8	72.2±4.7	6.5
	52.6	67.9±3.8	5.6
LUT	2.1	87.9±3.8	4.3
	103.5	82.1±4.0	4.8
	258.6	84.5±2.0	2.2

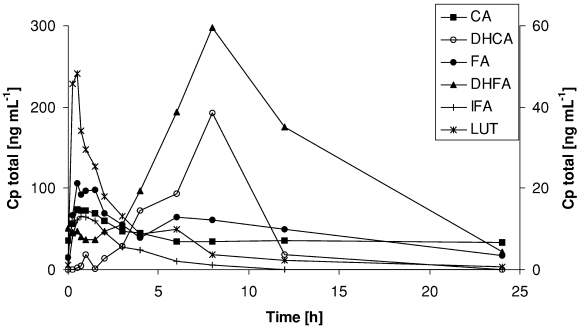

Fig. 2. Exemplary Cp time profiles (not fitted) of target compounds for one volunteer. Data for CA, DHCA, FA, DHFA and IFA correspond to the right *y*-axis. Data for LUT correspond to the left *y*-axis.

derived from artichoke leaf extract was developed. Due to the redox sensitivity of all target compounds, coulometric array detection was found to be an appropriate detection system for the highly sensitive and selective determination of polyphenols in a biological matrix [21,24,25]. LOQ values in the lower ng mL^{-1} range were achieved for all target compounds. Compared with other electrochemical systems used for plasma analysis [14,26,27] the multi-channel array detector had 12 electrodes in series set incrementally to different potentials. Thus the detector provided, for each of the target compounds, the detection potential providing the highest selectivity. For the separation of all target compounds from the plasma matrix, a solvent gradient was used. Including column flushing and equilibration, the HPLC analysis took 108 min. This is a rather long time, but it enables the simultaneous determination of six metabolites derived from artichoke leaf extract within one method. Comparable results with respect to sensitivity and selectivity towards hydroxycinnamates in human plasma have only been reported by Cremin et al. for a LC–ES-MS method. However, flavonoids were not considered and the method was not fully validated according to international guidelines for validation of bioanalytical methods. The limit of detection and calibration curves were obtained from standard solutions instead of spiked plasma samples. Moreover, data for the precision and accuracy of the method were lacking as only repeated recovery experiments were performed [28]. But LC–ES-MS analysis might

be a reasonable addition to this method in order to determine phase II conjugates such as glucuronides or sulphates of hydroxycinnamates or flavononoids.

In contrast to the long analysis time, the sample preparation procedure was simple and consisted only of a few steps. The method of Graefe et al. [22], developed for the determination of quercetin in human plasma, was modified in order to obtain a sufficient recovery of hydroxycinnamates and luteolin. To ensure total protonation of hydroxycinnamates, samples were additionally acidified and the final solvent was changed in order to increase the solubility of the target compounds. The previously described methods were only performed to extract single compounds such as caffeic acid or luteolin from a plasma matrix, and did not cover the range of hydroxycinnamates and flavonoids. Most used solid-phase extraction or liquid–liquid extraction, which included more steps than the method presented here. Although the recoveries obtained for caffeic acid extracted by liquid–liquid extraction were between 98.0 and 102.4% [19,27,28] and those for luteolin extracted by solid-phase extraction were 103.8% [16], our method ensures the simultaneous extraction of all target compounds in one extraction procedure with sufficiently reproducible recoveries of at least 62.2%. The difficulty of determining more than one analyte in the same extraction procedure is exemplified by a recent investigation of Cremin et al., who extracted caffeic acid, ferulic acid and chlorogenic acid simultaneously by liquid–liquid extraction and obtained recoveries in the range of 22.8–99.9% [28].

Due to the minor peaks observed for blank plasma treated with β-glucuronidase at the retention times of all target compounds except for isoferulic acid, for validation purposes sample preparation was performed without adding β-glucuronidase. All of the peaks were separated from proximate plasma peaks and were clearly identified from their hydrodynamic voltammograms. As there was no change in selectivity using blank plasma with or without adding enzyme, our approach is more authentic for validation purposes than quantifying compounds in human plasma by an external calibration performed in pure solvents.

Therefore, the developed method is regarded as being suitable for analyzing the bioavailability and pharmacokinetics of samples after oral administration of artichoke leaf extract. All validation criteria were in the range required by the international guidelines for validation of bioanalytical methods [23]. Linearity of the method was confirmed for all target compounds over the entire calibration range. Values for precision (RSD) and accuracy (RE) were ≤15% and at the LOQ ≤20% for all target compounds.

After oral administration of artichoke leaf extract to humans, each of the target compounds could be detected in plasma using this method. Thus, this is the first study showing the pharmacokinetic profiles of six metabolites derived from artichoke leaf extract in human plasma after β-glucuronidase treatment. Nardini et al. could only determine caffeic acid in plasma treated with β-glucuronidase after ingestion of coffee, but failed to detect other metabolites of chlorogenic acid [27]. In the only study of the metabolism of artichoke leaf extract, Rechner et al. demonstrated the presence of ferulic acid, isoferulic acid, dihydroferulic acid and vanilic acid in hydrolyzed urine, whereas, in human plasma, only trace amounts of ferulic acid could be detected. Luteolin was not present in urine or plasma [12].

As the six metabolites detected in human plasma after ingestion of artichoke leaf extract were most likely derived from different metabolic pathways, their C_{max} and t_{max} values differed. This is in agreement with data from Rechner et al. obtained after ingestion of coffee. After β-glucuronidase treatment, apart from unspecific benzoic acid derivatives, dihydroferulic acid was the most dominant metabolite excreted in human urine, with maximum amounts reached 8 to 12 h after coffee consumption. In contrast, ferulic acid and isoferulic acid were mainly excreted during the first 1–3 h and to a considerably smaller extent [29].

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