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High-performance liquid chromatographic assay for α-lipoic acid and five of its metabolites in human plasma and urine

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Abstract

An isocratic reversed-phase HPLC method for the simultaneous quantitation of α-lipoic acid and five of its metabolites in human plasma as well as in human urine employing solid-phase extraction and pulsed amperometric detection was developed and validated. The method was found to be sufficiently precise and accurate for the measurement of α-lipoic acid and its metabolites 6,8-bis(methylthio)octanoic acid, 4,6-bis(methylthio)hexanoic acid and 2,4-bis(methylthio)butanoic acid in plasma and urine samples, obtained from patients suffering from diabetic neuropathy as well as from healthy volunteers following daily oral administration of 600 mg α-lipoic acid. The quantitation of the metabolite bisnorlipoic acid was often impaired by interferences caused by an unidentified metabolite. However, bisnorlipoic acid was detected in few test samples and the concentrations were consistently low. Despite the poor recovery of the metabolite tetranorlipoic acid from plasma, reproducibility and accuracy were found to be from acceptable magnitude to assess concentration time courses. Thus, the obtained analytical results are considered as reliable and well suited for pharmacokinetic studies of α-lipoic acid and its metabolites.

Keywords: α-Lipoic acid

1. Introduction

McCormick et al. revealed the predominant metabolic pathway of α-lipoic acid (LA) in the rat, like in certain bacteria, mainly as β-oxidation of the valeric acid side chain and identified bisnorlipoic acid (BNLA), tetranorlipoic acid (TNLA) and β-hydroxybisnorlipoic acid in urine samples of rats [1,2]. Pharmacokinetic studies in man have been shown that LA undergoes extensive first-pass metabolism [3,4]. Moreover, the enantioselective pharmacokinetics and bioavailability of the LA enantiomers in man have been extensively investigated [3]. In a preliminary study as major metabolite, 4,6-bis-(methylthio)hexanoic acid (BMHA) has been found in urine of healthy volunteers, but only small quantities of 6,8-bis(methylthio)octanoic acid (BMOA) and 2,4-bis(methylthio)butanoic acid (BMBA) have been detected [5]. LA was only a minor component in the urine. Furthermore, BMHA has been detected as the predominant circulating metabolite following oral administration of LA [6]. Recently, the metabolic pathways of LA in mice, rats, dogs and humans were investigated by means of radiometric HPLC and LC/tandem mass spectroscopy [7]. However, no paper has been published so far neither on the time

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course of any LA metabolite concentrations in plasma nor on urinary excreted amounts.

Various methods have been developed for the determination of LA and related compounds. An overview of chromatographic methods has been published by Kataoka [8]. However, until now, less attention has been directed to the quantitative analysis of LA metabolites in man.

Although pulsed electrochemical detection (PED) at noble metal electrodes has gained prominence for the oxide-free determination of carbohydrates and related analogues, Owens and LaCourse [9] described it as a useful method for the simple, sensitive and direct determination of thiols, mono- and disulfides at a single electrode. Johnson and LaCourse [10] have suggested to use the generic title PED, which includes the techniques pulsed amperometric detection (PAD) and integrated pulsed amperometric detection (IPAD), for all detection strategies based on the application of multistep waveforms. It must be emphasized that sulfur-containing compounds are considered to be strongly adsorbed to the electrode surface. Conventional DC electrochemical detection has not been considered applicable for quantitation of most sulfur compounds, because of the observed loss of electrode activity due to the accumulation of sulfurous adsorbates. However, we measured LA with high sensitivity using glassy-carbon electrode amperometric detection, at the high oxidation potential of 1.1 V [11]. Despite regular cleaning of the electrode surface, we observed an extreme loss of electrode sensitivity regarding the monosulfidic LA metabolites in a short period. Hence, a method with PAD has been developed which shows adequate sensitivity for disulfidic as well as for monosulfidic metabolites over a broad concentration range. The method is direct, i.e. derivatization is not required and employs simple solid-phase extraction (SPE). Therefore, it is well suited for investigations concerning both LA and its metabolites in biological materials.

2. Experimental

2.1. Apparatus and chromatography

For sample extraction, a Gilson Medical Electronics S.A. ASPEC XL (Villiers-le Bel, France) automated solid-phase extraction workstation was used. Chromatography was performed using a Knauer (Berlin, Germany) WellChrom Mini-Star K-500 pump. Detection was performed with a Thermo Separation Products (Riviera Beach, FL, USA) electrochemical detector EC2000 equipped with an EC2000 standard flowcell (Ag/AgCl reference electrode, gold working electrode diameter 3 mm, flowcell volume 0.35 μl, pulse mode). PAD pulse settings were E_1=1.2 V, E_2=1.4 V, E_3=−0.6 V, t_1=650 ms, t_2=550 ms, t_3=70 ms and t_s (sampling time)=20 ms. Samples were injected manually using a Rheodyne 7725i injector. The separations were accomplished with an Ultrasep ES PHARM RP18, 5 μm, 150×3 mm analytical column equipped with a precolumn, 10×3 mm (SepServ, Berlin, Germany) by isocratic elution at a flow-rate of 0.55 ml/min. The columns and the electrochemical cell were temperature controlled at 30 °C in the Faraday-shielded oven compartment of the detector. The mobile phase consisted of 50 m*M* KH_2PO_4 (pH adjusted to 2.3 with H_3PO_4) -acetonitrile (69:31, v/v) degassed with helium prior to use. Data were collected via computer with Knauer HPLC Software/Hardware Package version 2.11.

2.2. Chemicals and reagents

Racemic LA and the five hypothetic degradation products (BMOA, BMHA, BMBA, BNLA and TNLA) were kindly provided by ASTA Medica AG (Frankfurt am Main, Germany), AWD Arzneimittelwerk Dresden GmbH (Radebeul, Germany). The purity of the crystalline substances LA, BNLA and TNLA was >98%. BMOA, BMHA and BMBA were viscous oils with a certified purity of 96.4, 88.6 and 93.0%, respectively. Acetonitrile (ultra gradient grade), water (HPLC grade) and potassium dihydrogen phosphate (ultrapure bioreagent grade) were purchased from J.T. Baker (Deventer, The Netherlands). All other chemicals were analytical grade, and were purchased from Merck (Darmstadt, Germany) or from Sigma (Taufkirchen, Germany). β-Glucuronidase/aryl sulfatase (from Helix pomatia) stabilized aqueous solution for biochemistry was purchased from Merck. BAKERBOND spe Phenyl (1 ml/100 mg, Product No. 7095-01) extraction columns were purchased from J.T. Baker. Human plasma with sodium heparine (50 U/ml, Liquemin®

N 10 000, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) as anticoagulant was obtained from numerous blood donors.

2.3. Plasma sample preparation

Samples and quality controls were processed in batch. Conditioning was carried out by passing 1 ml of methanol through the cartridge. Equilibration was achieved by passing through 1 ml of water, pH 3.0. An aliquot of 3 ml of water and 10 μl of concentrated HCl were added to 0.5 ml of plasma, which was loaded with a flow-rate of 1 ml/min onto the equilibrated cartridge. The remaining proteins were washed from the column with 2 ml of water, pH 3.0. Then, 5 ml of air were drawn through the column to dry it slightly. Elution of the analytes was achieved with 1 ml of dichloromethane at a flow-rate of 1 ml/min. The eluate was collected into a glass tube, and evaporated under nitrogen at 40 °C. The extraction residues were reconstituted in 0.2 ml of mobile phase. A total of 20-μl aliquots of the resulting solutions were injected onto the HPLC column for analysis. If the working electrode was overload due to the high concentration of an analyte, the reconstituted samples were further diluted with mobile phase and reanalyzed.

2.4. Urine sample preparation

Samples and quality controls were processed in batch. To 0.1 ml urine, 0.1 ml of citrate–phosphate buffer (pH 5.5) was added containing 1 μl glucuronidase/arylsulfatase. The mixture was heated at 55 °C for 15 min followed by addition of 3.3 ml of water containing 10 μl of concentrated HCl. The SPE column was conditioned by passing through 1 ml of methanol followed by 1 ml of water, pH 3.0. The column was not allowed to dry before sample application. The 3.5-ml volume of sample was applied to the column within 3.5 min, followed by rinsing with 0.1 ml of water, pH 3.0. The column was dried by passing through 5 ml of air with a flow-rate of 80 ml/min. The analytes were eluted with 2 ml of dichloromethane. The eluate was then dried under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 400 μl of mobile phase, and a 20-μl aliquot was injected onto the HPLC column. Depending on the concentrations of the analytes the reconstituted samples were further diluted with mobile phase.

2.5. Standard solutions

Stock solutions of LA and the five metabolites (Fig. 1) were prepared by dissolving the reference substances in acetonitrile to a final concentration of 10.0 μmol/ml (LA, BNLA, TNLA), 944.4 μmol/ml (BMOA), 442.1 μmol/ml (BMHA) and 218.5 μmol/ml (BMBA). Aliquots of stock solutions of BMOA, BMHA and BMBA (0.5 ml each) were found to be stable at ≤ -65 °C for at least 2 years. Stock solutions of LA, BNLA and TNLA were freshly prepared quarterly from the crystalline pure substances and stored on the same way. Working solutions for the preparation of urine standards were obtained by appropriate dilution of the stock solutions with acetonitrile. For the preparation of plasma standards, the stock solutions were diluted appropriately to give standard solutions in water (pH 8).

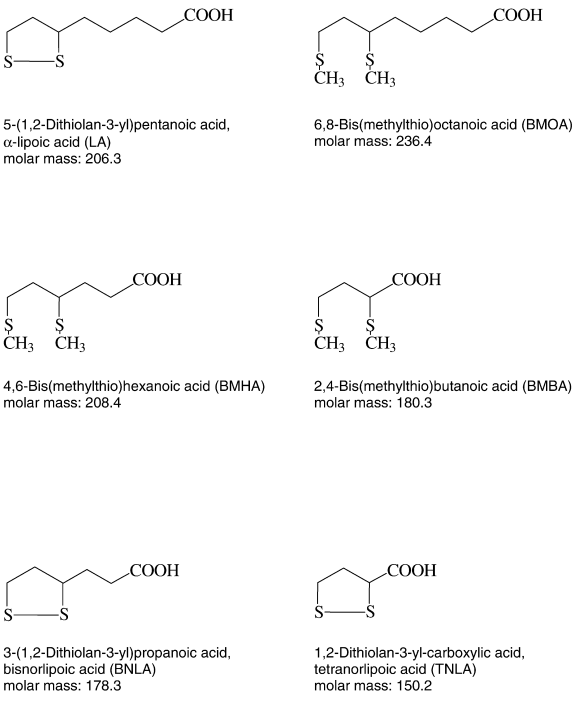

Fig. 1. Chemical structures of LA and its five metabolites.

2.6. Samples for pharmacokinetic study

Analyses were performed using plasma and urine samples from healthy volunteers and patients suffering from renal impairment following repeated single dose administration of 600 mg racemic LA over 4 days. Blood samples for PK evaluation were taken at study day 1 and study day 4 up to 12 h after dosing and every study day before dosing. Serial blood samples were collected in heparinized Monovette plastic tubes (Sarstedt, Nümbrecht, Germany) from cubital vein. The samples were centrifuged immediately following withdrawal of blood (1500 *g*, 5 min). The supernatant plasma was transferred to clean tubes, and stored in a frozen state at ≤ -65 °C until time of analysis. Urine samples for the test drug assay were collected in Sarstedt Monovette plastic tubes (10 ml Urine-Monovette®, Sarstedt, Nümbrecht, Germany) up to 24 h after dosing at study day 1 and up to 48 h after dosing at study day 4 and stored in a frozen state at ≤ -65 °C until time of analysis.

2.7. Method validation

Calibration curves for six analytes were constructed by analyzing control plasma and urine spiked with the standards of LA and its metabolites. Since reproducibility was dependent on the value of measurement (heteroskedastic system), weighted ($s_i^{-2}/\Sigma s_i^{-2}/m$, s_i = standard deviation of each value, m = number of measured concentration levels) linear least squares regression was used to assess linearity of the calibration curves. Peak areas or peak heights versus the nominal concentrations were used to generate the curves. The concentrations of plasma and urine analytes were determined from the calibration curves. For in-study control, at least three QC samples were co-analyzed within one HPLC batch. Depending on the sample size a maximum of six QC samples were co-analyzed. Typically, the deviation was less than 2 SD (warning region) as determined from the measurements of QC samples prepared at low, medium, and high concentrations within the range of the calibration curves. One of the three or two of the six QC samples could be outside the ± 3 SD nominal value (control region) if both are not of the same concentration. Otherwise the results of the corresponding batch were not accepted and the test samples had to be reanalyzed.

The selectivity was assessed by analyzing the control blank plasma and urine spiked with the reference standards as well as several test samples obtained from a renally impaired patient. Analysis of the authentic reference standards of LA and its metabolites was conducted to identify the retention times of the analytes. The lack of interfering peaks at the same analyte retention times was considered as acceptable selectivity.

In general, the assay precision and accuracy were derived from the calibration samples which were quantitated with the calibration curves. From this data, the mean, standard deviation and coefficient of variation at each level were determined. The accuracy of the method was calculated by comparing the results obtained with those of the expected theoretical concentration. The within-day reproducibility and accuracy were calculated by analyzing QC samples at the low, medium, and high concentration level in six replicates. The analyses for calculation of between-day accuracy and reproducibility were performed on 6 days.

Samples containing at least three different concentrations of the analytes (low, medium and high level) were used for the determination of recoveries of LA and the metabolites. Recoveries were calculated as the mean of six replicates of both spiked samples and standard solutions at each concentration. The detector response of extracted samples was compared with the detector response of standard solutions containing equivalent amounts of LA and the metabolites, respectively.

To examine the stability in plasma and urine, pools of blank plasma and urine were spiked with the reference substances to obtain high, medium and low concentrations. These QC-spiked plasma and urine pools were pipetted into aliquots and stored under the same conditions (≤ -65 °C) as the routine samples. Analyses were performed after 4, 7 and 8 months (urine samples) and after 0.5, 1, 2, 3, 4 and 12 months (plasma samples).

Numerous substances were tested for the purpose of use as internal standard. For this reason, various substances were synthesized, which contain a methylthio as well as a carboxyl group. No substance was found, which was suitable as internal standard.

Therefore, in this study the method of external standard was used.

3. Results and discussion

For sulfur compounds PED occurs with simultaneous formation of surface oxide. The anodic detection mechanism is believed to involve prior adsorption of compounds at the oxide-free electrode surface. The concomitant formation of surface oxides catalyzes the oxidation of sulfur atoms. A disadvantage of PED is the large anodic background current and the fact that the kinetics of surface oxide formation can be influenced by the adsorbed analyte. The calibration curve is strongly influenced by the absorption isotherm of the analyte and therefore, it deviates from linearity at high concentrations. However, due to the high concentrations appearing after administration of therapeutic doses, the method must cover a broad concentration range. In order to minimize the influence of the adsorbed analyte we performed all measurements in the same detector range of 0.1 μA. Due to the varying concentrations of the analytes, most of the samples had to be diluted differentially and were analyzed repeatedly. The dilution factor possessed a certain influence on peak sensitivity and therefore, it was included in the calibration procedure. Hence, not the quantity injected into the chromatographic system, but the real concentration in the sample was plotted versus peak area and peak height, respectively. The extracted samples were diluted in the same manner as the calibrators.

The structural identity of the metabolites in both plasma and urine was confirmed by comparing their retention times and their LC–MS profile with those of previously characterized reference compounds.

3.1. Plasma

As detailed in this report, the assay was reproducible for LA and its metabolites, as judged generally by a coefficient of variation of less than 15%. The plasma standard curves were linear over the following concentration ranges: 0.05–50.0 nmol/ml (LA), 0.25–5.0 nmol/ml (BMOA), 0.25–50.0 nmol/ml (BMHA), 0.25–25.0 nmol/ml (BMBA), 0.10–35.0 nmol/ml (BNLA), 0.2–80.7 nmol/ml (TNLA). For each point of calibration standards, the concentrations were back-calculated from the equation of the linear weighted regression curves. Typical calibration curves had the regression equation of $y = 9778.297x + 45.889$ (LA, by means of peak area) and $y = 250.292x + 3.293$ (TNLA, by means of peak height) with coefficients of correlation (r) of 0.999 and 0.998, respectively. The recoveries of LA and the metabolites following SPE of spiked plasma are presented in Table 1. Due to the strong polarity a predominant part of the TNLA moiety passed through the column resulting in poor recovery. However, reproducibility and accuracy were found to be from acceptable magnitude to assess concentration time courses of TNLA. A summary of the results of the intra- and inter-assay precision for LA and the metabolites is given in Table 2. The proposed limit of quantitation, i.e. $LOQ = 6\sigma/m$, were estimated based on the standard deviation σ of the

Table 1
Recovery of LA and the metabolites ($n = 6$)

	LA	BMOA	BMHA	BMBA	BNLA	TNLA
Plasma						
$Mean_{ar}$ (%)	90.0	75.6	85.6	78.4	83.4	10.4
SD (%)	3.2	3.4	3.4	4.4	6.3	0.9
m	10	6	10	10	9	9
Urine						
$Mean_{ar}$ (%)	82.5	82.7	81.4	79.7	81.4	44.9
SD (%)	5.4	1.3	2.9	1.4	2.2	2.6
m	3	3	3	3	3	3

m = number of concentration levels, $Mean_{ar}$ = arithmetic mean.

Table 2
Precision and accuracy data of the determination of LA and its metabolites quantitated with the weighted linear regression

	LA	BMOA	BMHA	BMBA	BNLA	TNLA
Plasma intra-day						
C.V. (%)	3.4–6.5	3.1–4.9	2.0–6.6	0.8–6.5	3.8–5.5	5.4–7.5
$Mean_{ar}$±SD	4.9±1.2	3.8±0.9	4.3±1.7	3.4±2.0	4.4±1.0	6.4±1.0
Accuracy (%)	95.7–106.5	99.2–105.5	95.8–102.1	96.0–109.8	85.3–101.5	102.4–126.8
$Mean_{ar}$±SD	101.0±3.8	102.0±2.7	97.8±2.6	101.9±5.4	94.9±8.5	111.2±13.5
m	6	6	6	6	6	6
Plasma inter-day						
C.V. (%)	1.9–6.7	4.8–7.1	3.0–7.2	3.5–8.0	5.1–17.8	3.7–14.2
$Mean_{ar}$±SD	3.9±1.8	5.7±1.0	4.7±1.6	5.9±1.7	10.4±6.6	7.6±5.3
Accuracy (%)	99.6–104.5	99.5–102.1	97.5–102.9	95.4–105.8	90.2–100.6	97.8–104.0
$Mean_{ar}$±SD	100.9±1.8	99.8±2.3	99.3±1.9	99.1±3.4	94.2±5.5	100.6±3.1
m	10	6	10	10	9	9
Urine intra-day						
C.V. (%)	6.4–10.6	6.9–11.1	6.3–9.0	5.2–8.2	5.9–11.9	3.7–12.1
Mean±SD	8.3±2.1	8.4±2.3	7.9±1.4	6.2±1.7	8.0±3.3	6.8±4.6
Accuracy (%)	102.0–102.7	94.8–110.0	96.4–109.7	91.5–101.3	94.1–94.9	90.2–104.4
$Mean_{ar}$±SD	102.5±0.4	101.9±7.7	101.0±7.6	97.2±5.1	94.5±0.4	96.4±7.3
m	3	3	3	3	3	3
Urine inter-day						
C.V. (%)	3.8–13.0	8.6–12.0	7.1–8.0	6.4–9.4	4.0–9.6	9.5–9.7
$Mean_{ar}$±SD	7.4±4.9	9.7±2.0	7.5±0.4	8.0±1.5	6.6±2.8	9.6±0.1
Accuracy (%)	98.4–110.5	97.3–103.7	94.0–107.2	98.2–101.0	90.9–101.8	101.7–110.6
$Mean_{ar}$±SD	105.4±6.3	100.6±3.2	99.0±7.2	99.2±1.6	97.5±5.8	106.2±4.4
m	7	7	7	7	7	7

m = number of concentration levels (number of replicates = 6), $Mean_{ar}$ = arithmetic mean.

peak response and the slope m of the calibration curve. The LOQs calculated by linear regression were 0.022 nmol/ml for LA, 0.15 nmol/ml for BMOA, 0.15 nmol/ml for BMHA, 0.08 nmol/ml for BMBA, 0.084 nmol/ml for BNLA, and 0.072 nmol/ml for TNLA.

If BNLA appeared, BMBA was quantitated by means of peak height instead of peak area. At ≤ −65 °C the concentrations were found to be unchanged for at least 13 months, ranging from −5.1 to 8.9% compared to analysis of samples without previous exposure to freezing.

The chromatograms of various blank plasma samples and those of plasma containing the reference substances showed sufficient separation from the matrix constituents. No endogenous components interfered significantly with either LA or LA metabolites peaks. The separation of BMBA from BNLA was difficult with a resolution (R_s) of ≤1.0. However, BNLA only appeared in a short period and the concentrations were consistently low. Moreover, BNLA was completely lacking in several samples. When BMBA appeared, it was quantitated by means of peak height instead of peak area. Furthermore, interferences impairing the quantitation of BNLA caused by an unidentified metabolite could be observed in some test samples (Fig. 2B). In Fig. 2 typical chromatograms are shown obtained after processing 0.5 ml plasma spiked with 208 ng LA, 443 ng BMOA, 521 ng BMHA, 316 ng BMBA, 72 ng BNLA and 307 ng TNLA (Fig. 2A), after processing plasma from a healthy volunteer (Fig. 2B) as well as a renally impaired patient (Fig. 2C), both treated with a single daily dose of 600 mg LA.

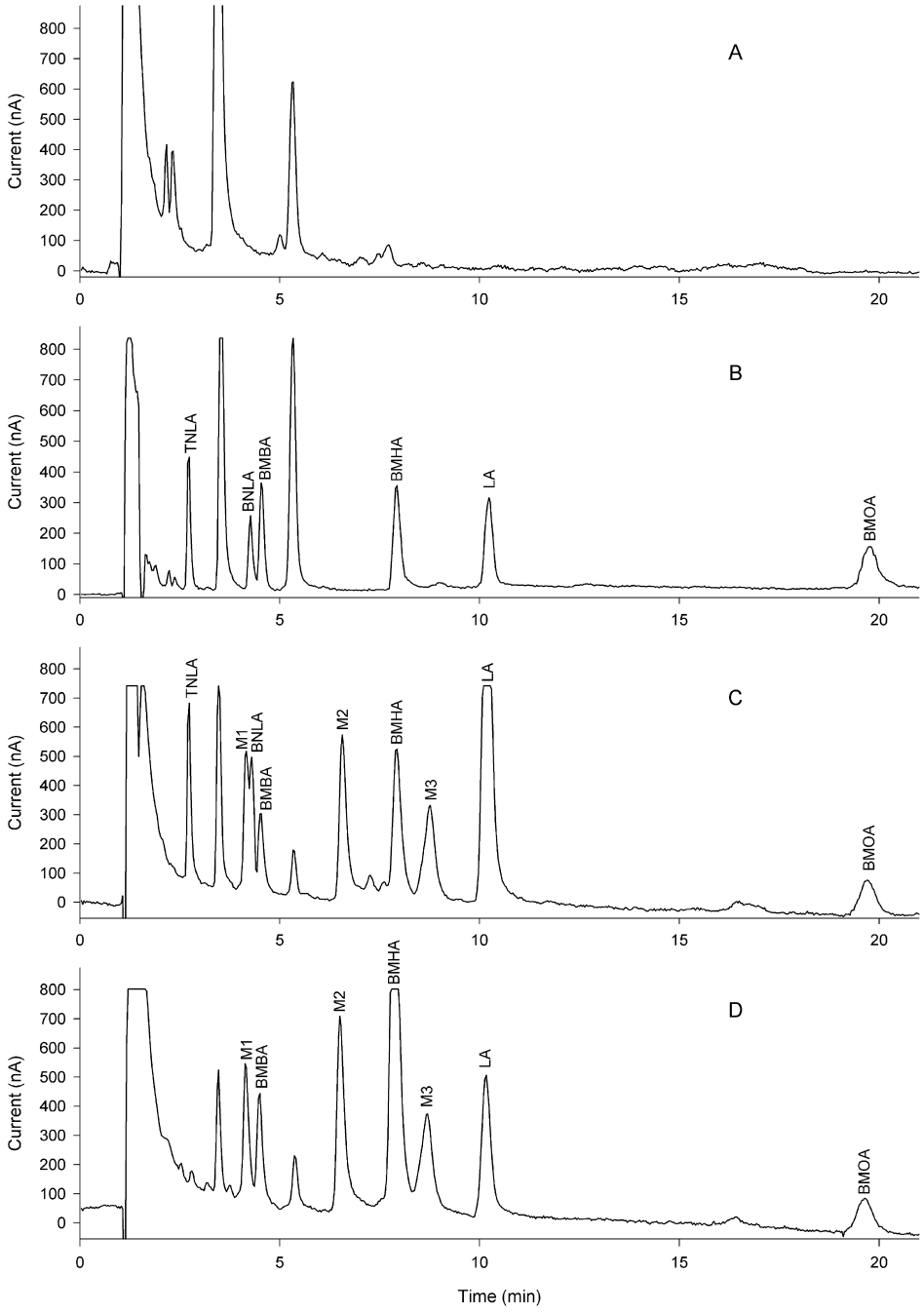

Fig. 2. Typical chromatograms of human plasma extracts. (A) Blank plasma (undiluted). (B) Blank plasma per ml spiked with 2 nmol LA, 3.75 nmol BMOA, 5 nmol BMHA, 3.5 nmol BMBA, 0.4 nmol BNLA and 4.03 nmol TNLA (3× diluted). (C) Plasma of a healthy volunteer 45 min after a single oral dose of 600 mg LA containing 13.06 nmol LA, 2.62 nmol BMOA, 7.08 nmol BMHA, 2.78 nmol BMBA, 0.92 nmol BNLA and 12.57 nmol TNLA per ml (5× diluted). For quantitation of LA the 20-fold diluted extract was injected. (D) Plasma sample obtained from a patient 75 min after a single oral dose of 600 mg LA containing 3.93 nmol LA, 2.38 nmol BMOA, 19.22 nmol BMHA, 3.82 nmol BMBA and 1.23 nmol TNLA per ml plasma (5× diluted). BMHA was quantitated after 20-fold dilution. The peaks M1, M2 and M3 represent unidentified metabolites.

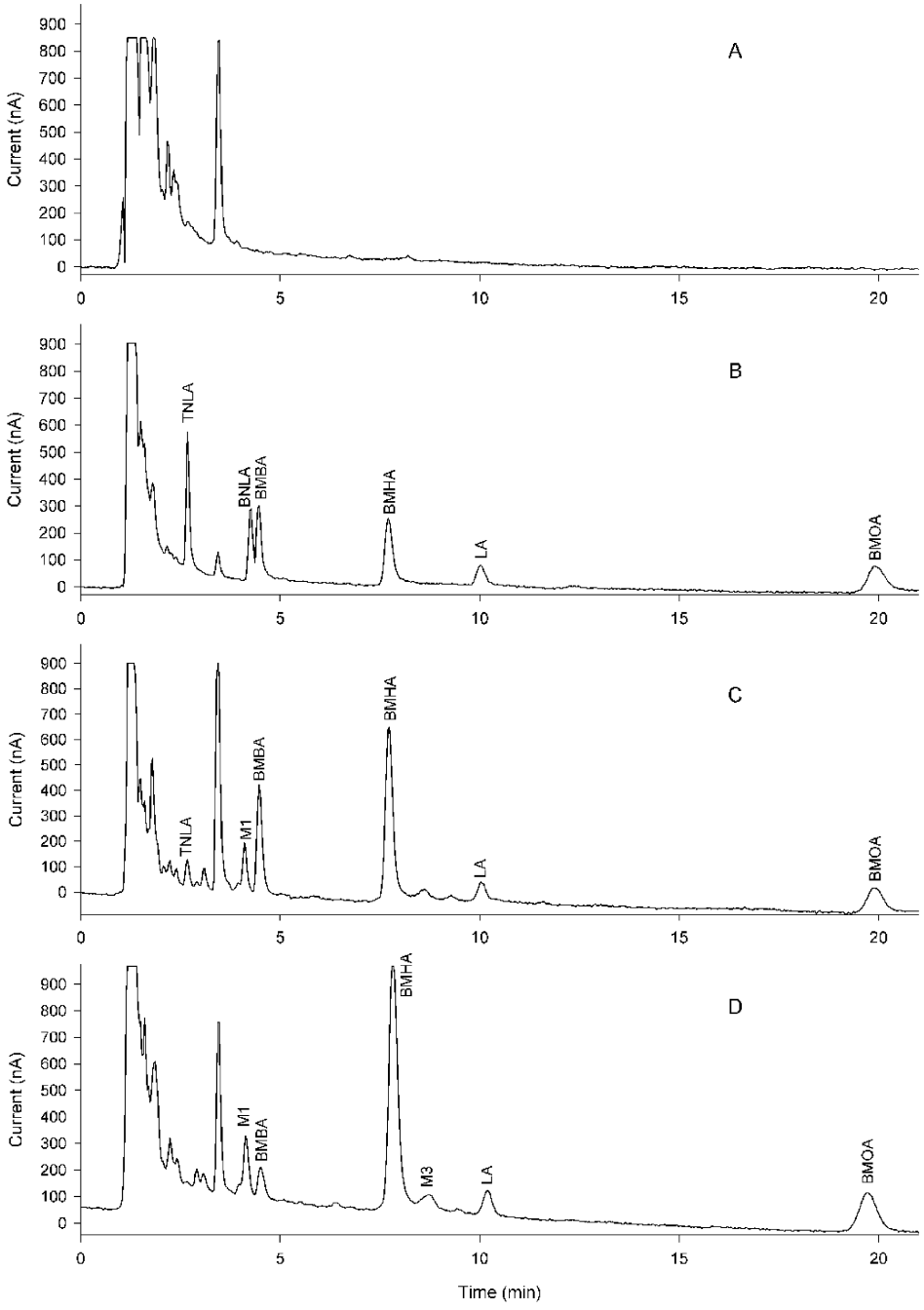

Fig. 3. Chromatograms of LA and metabolites in urine extracts. (A) Blank urine (5× diluted). (B) Blank urine per ml spiked with 7.5 nmol LA, 30 nmol BMOA, 50 nmol BMHA, 35 nmol BMBA, 15 nmol BNLA and 25 nmol TNLA (10× diluted). (B) Urine of the same healthy volunteer as depicted in Fig. 2B containing 11.7 nmol LA, 41.6 nmol BMOA, 196.3 nmol BMHA, 89.9 nmol BMBA and 10.3 nmol TNLA per ml (20× diluted). (C) Urine of the patient depicted in Fig. 2C containing 6.4 nmol LA, 68.6 nmol BMOA, 470.4 nmol BMHA and 25.4 nmol BMBA per ml (5× diluted).

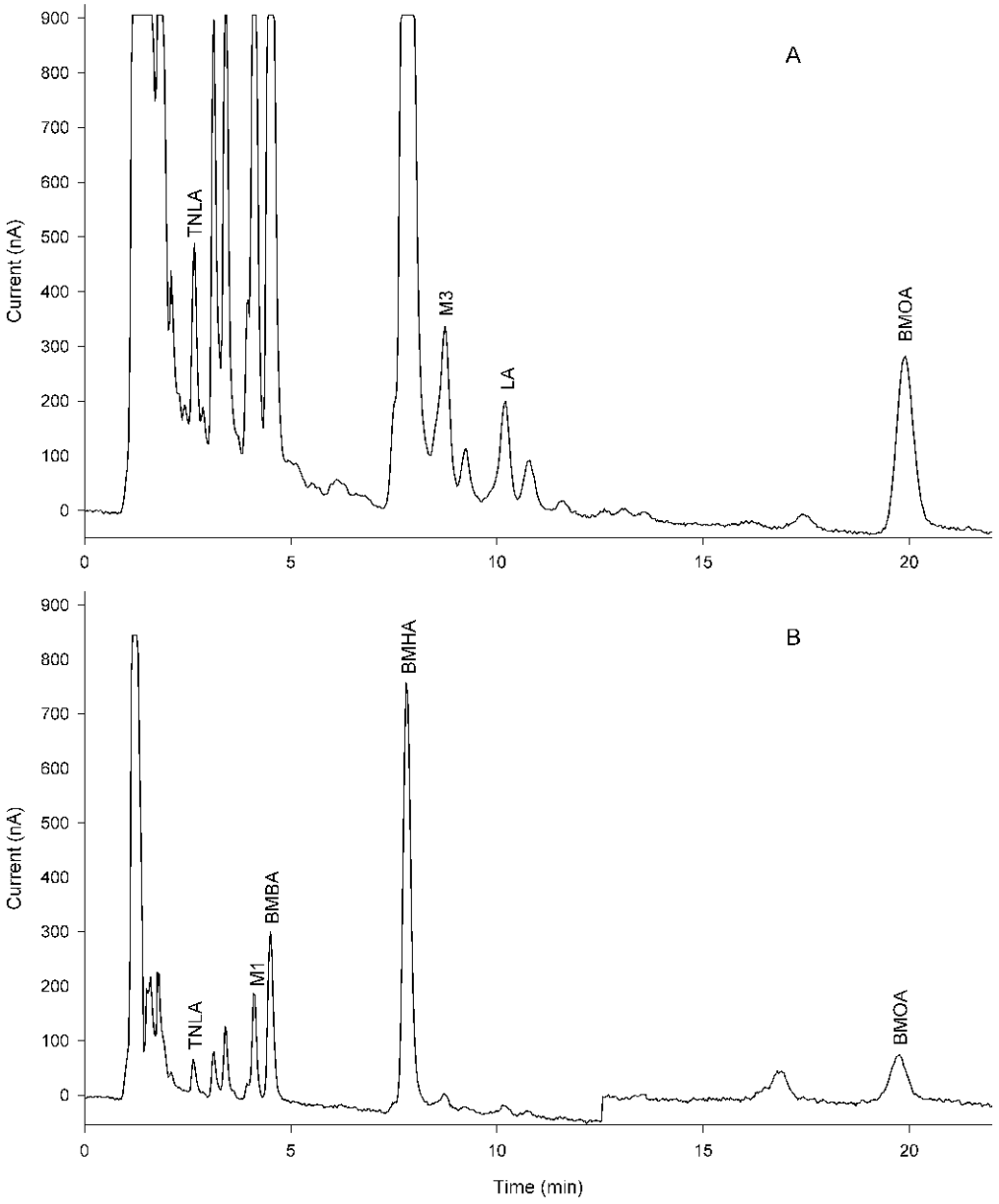

Fig. 4. Chromatograms of an urine extract reconstituted in 200 μl of mobile phase after 10-fold dilution for quantitation of TNLA, LA and BMOA (A), and after 100-fold dilution to quantitate BMBA and BMHA (B).

3.2. Urine

The urine standard curves were linear over the following concentration ranges: 0.75–150 nmol/ml (LA), 3–500 nmol/ml (BMOA), 2–5000 nmol/ml (BMHA), 2–750 nmol/ml (BMBA), 1–250 nmol/ml (BNLA), 2–500 nmol/ml (TNLA). The LOQs were 0.5 nmol/ml for LA, 1.48 nmol/ml for BMOA, 1.6 nmol/ml for BMHA, 0.92 nmol/ml for BMBA, 0.44 nmol/ml for BNLA, and 1.12 nmol/ml for TNLA. Typical calibration curves had the regression equation of $y = 1540.04x - 64.98$ (LA, peak areas) and

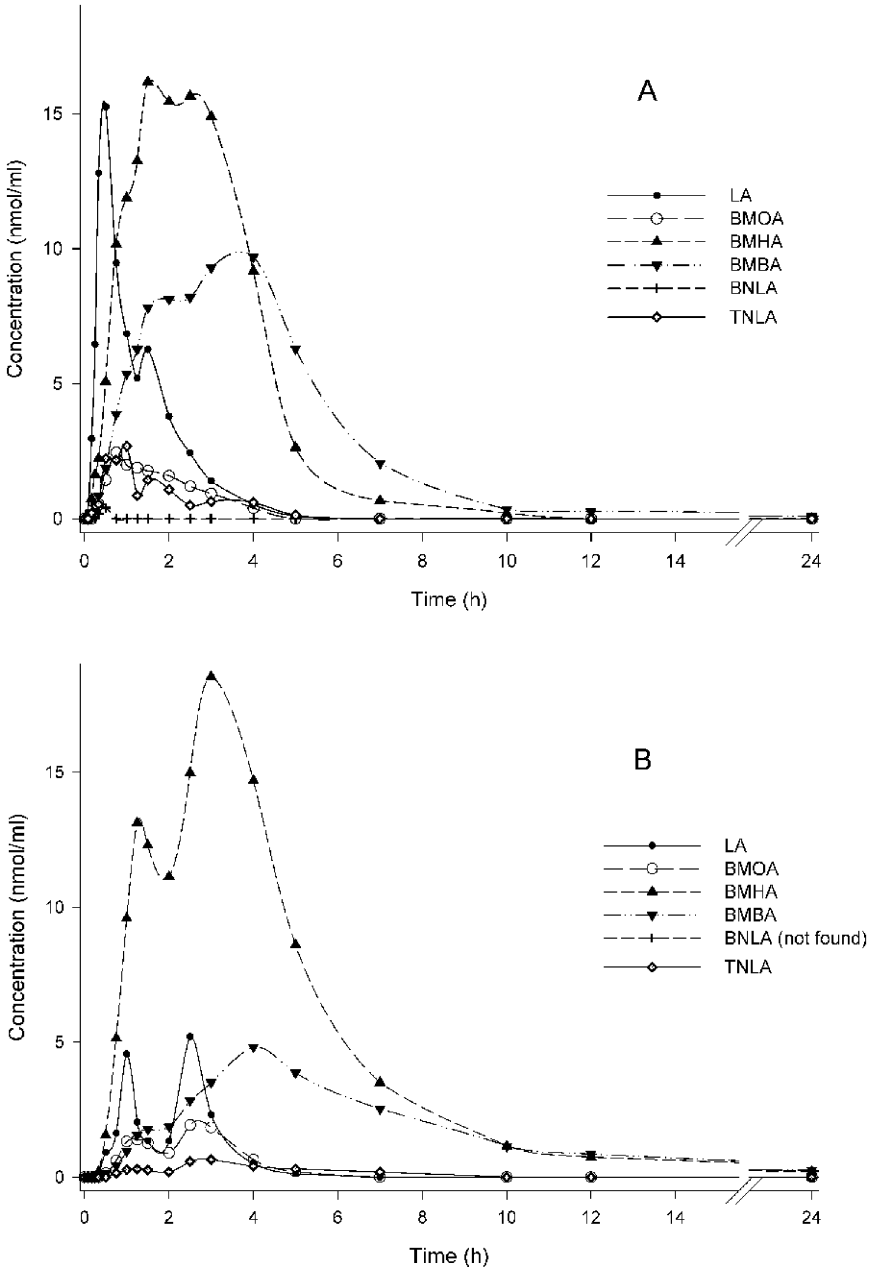

Fig. 5. Plasma concentration time courses of LA and its metabolites in the same volunteer (A) and patient (B) as depicted before, after a 600 mg oral dose of LA. BNLA was not found in the patient's sample.

$y = 69.199x - 12.416$ (BMBA, peak heights) with coefficients of correlation (r) of 0.9823 and 0.9955, respectively. The recoveries of LA and its metabolites following SPE of spiked urine samples are presented in Table 1. The recovery of TNLA from urine was significantly increased compared to those

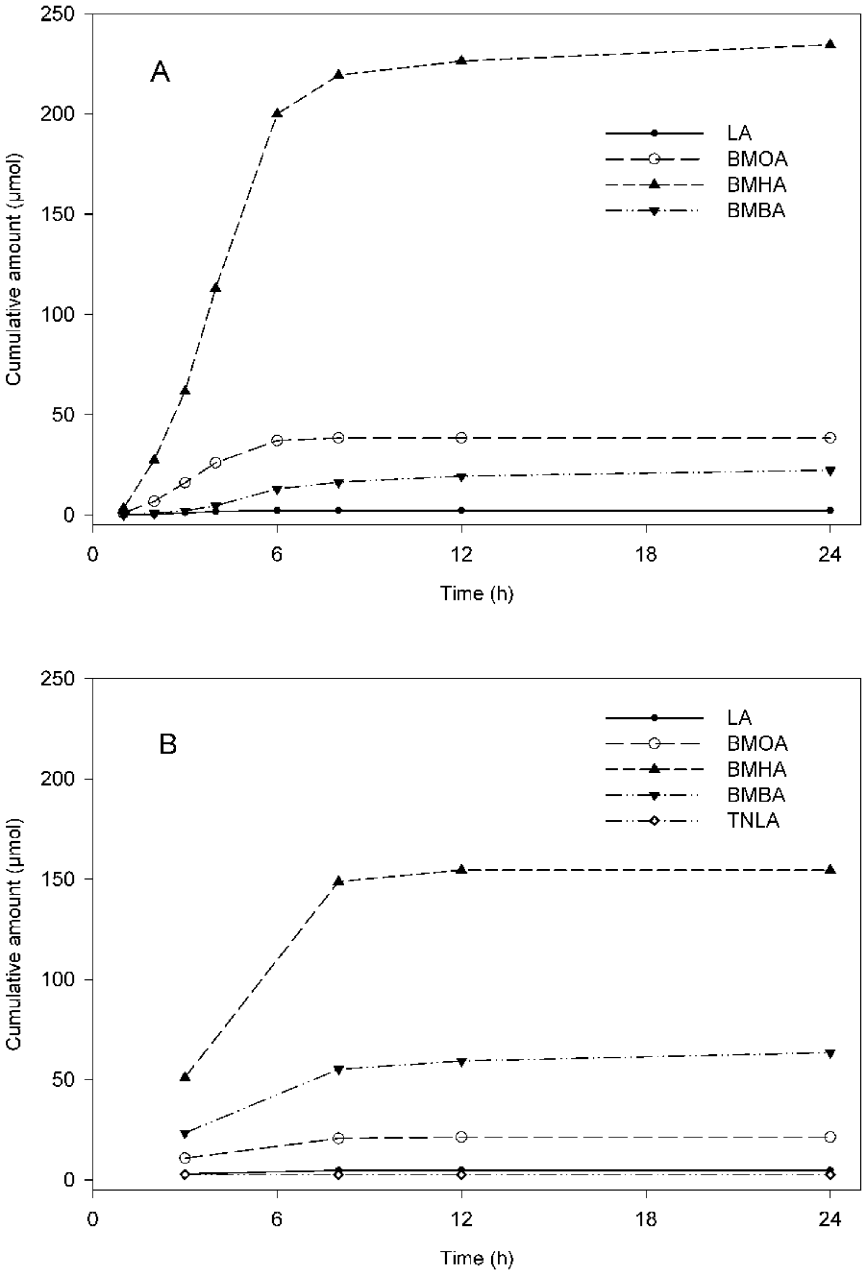

Fig. 6. Cumulative excretion of LA and metabolites in urine after a 600-mg oral administration to the healthy volunteer (A) and to the renally impaired patient (B) mentioned in all diagrams.

from plasma samples. The within-day reproducibility and the day-to-day reproducibility are shown in Table 2.

At $\leq -65\,^{\circ}C$ all compounds exhibited acceptable storage stability in human urine for at least 8 months, ranging from −4.0 to 7.5% compared to fresh prepared samples.

The peaks representing BMBA and BNLA ap-

peared closely to each other ($R_s \leq 1.0$) and were quantitated by means of peak height. However, BNLA was not often detectable in the urine test samples. Fig. 3 shows a typical chromatogram obtained after processing 0.1 ml urine spiked with 155 ng LA, 709 ng BMOA, 1042 ng BMHA, 631 ng BMBA, 267 ng BNLA and 380 ng TNLA (Fig. 3A), the levels of unchanged LA as well as LA metabolites in urine from the same volunteer (Fig. 3B) and from the same patient (Fig. 3C) as mentioned in Section 3.1 In Fig. 4 chromatograms are presented as example for the repeated measurement after different dilution in order to quantitate all substances.

3.3. Application to pharmacokinetic study

The described assay was successfully applied to measure plasma and urinary levels of LA and its metabolites collected from healthy volunteers, as well as from renally impaired patients participating in a open study, in order to document the pharmacokinetics of LA and its metabolites and to study renal elimination in this population. Pharmacokinetic parameters were determined using standard non-compartmental methods by means of the PK software TopFit [12]. Fig. 5 depicts the plasma versus time profiles of LA and its metabolites of a representative volunteer. Results indicated that BMHA and BMBA were the two major metabolites of LA in man, whereas BNLA concentrations were consistently low and could not be quantitated in numerous samples. Preliminary evaluation of the results indicate that less than 20% of the administered dose was excreted in the urine during the first 24 h after oral administration of 600 mg LA. Fig. 6 shows representative cumulative excretion profiles in the urine of LA and its metabolites in the 24 h after drug administration. Results from the pharmacokinetic analysis of these data will be published elsewhere.

4. Conclusions

The advantage of the method reported here is being the first HPLC assay with which LA and its metabolites can be quantitated simultaneously in biological samples after therapeutic administration of LA. Sample preparation is quite simple without derivatization procedure. Compared to manual SPE the automated SPE procedure used in this study has been provided improved recovery in particular for more polar compounds as well as superior precision.

Some test samples were analyzed prior to validation and neither BNLA nor TNLA were found in this samples of patients who received LA. However, during routine analysis BNLA and TNLA were detected beside other unidentified metabolites in several test samples. Therefore, chromatographic resolution of the more polar metabolites of LA should be improved by further development of this method. The well known disadvantages of PAD, drifting baselines attributable to variations in the extent of surface oxide formation, noisy baselines, higher limits of detection and postpeak ‘‘dips’’, are overcome performing IPAD waveform that requires more expensive equipment. Nevertheless, both PAD and IPAD are capable of direct and sensitive determination of sulfur-containing compounds. Although the presented method can not be applied to the measurement of endogenous LA levels which are in the range of 1 ng/ml (4.85 pmol/ml) or below, the method possesses adequate sensitivity, selectivity, accuracy, and precision for the quantitative analysis of LA and its metabolites and therefore, it is well applicable for the assessment of pharmacokinetics of exogenous LA and its major metabolites.

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References

[1] E.H. Harrison, D.B. McCormick, Biochem. Biophys. 160 (1974) 514.
[2] J.T. Spence, D.B. McCormick, Arch. Biochem. Biophys. 174 (1976) 13.
[3] R. Hermann, G. Niebch, H.O. Borbe, H. Fieger-Büschges, P. Ruus, H. Nowak, H. Riethmüller-Winzen, M. Peukert, H. Blume, Eur. J. Pharm. Sci. 4 (1996) 167.

[4] J. Teichert, J. Kern, H.-J. Tritschler, H. Ulrich, R. Preiß, Int. J. Clin. Pharmacol. Ther. 36 (1998) 625.
[5] M. Locher, E. Busker, H.O. Borbe, Naunyn Schmiedebergs Arch. Pharmacol. 351 (1995) R52.
[6] J. Teichert, R. Preiss, Naunyn Schmiedebergs Arch. Pharmacol. 361 (2000) R126.
[7] H. Schupke, R. Hempel, G. Peter, R. Hermann, K. Wessel, J. Engel, T. Kronbach, Drug Metabol. Dispos. 29 (2001) 855.
[8] H. Kataoka, J. Chromatogr. B 717 (1998) 247.
[9] W.R. LaCourse, G.S. Owens, Anal. Chim. Acta 307 (1995) 301.
[10] D.C. Johnson, W.R. LaCourse, Electroanalysis 4 (1992) 367.
[11] J. Teichert, R. Preiss, J. Chromatogr. B 672 (1995) 277.
[12] G. Heinzel, R. Woloszczak, P. Thomann, Topfit Pharmacokinetic and Pharmacodynamic Data Analysis System for the PC Version 2.0, Gustav Fischer, Stuttgart, Jena, New York, 1993.