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Journal of Chromatography B, 767 (2002) 93–101

JOURNAL OF CHROMATOGRAPHY B

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Determination of alachlor and its metabolites in rat plasma and urine by liquid chromatography–electrospray ionization mass spectrometry

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Received 28 June 2001; received in revised form 31 October 2001; accepted 5 November 2001

Abstract

A method based on liquid chromatography (LC) in combination with mass spectrometry (MS) for the analysis of alachlor (ALA) and its metabolites, 2-chloro-*N*-[2,6-diethylphenyl]acetamide (CDEPA) and 2,6-diethylaniline (DEA), in rat plasma and urine has been developed. ^{13}C-labeled ALA was used as the internal standard for quantitation. The analyte in plasma or urine was isolated using a Waters Oasis HLB extraction plate. The mass spectrometer was operated in the ESI MS-SIM mode with a programming procedure. The retention times for ALA, CDEPA and DEA were 1.84, 3.11 and 4.12 min, respectively. The limits of quantification (LOQ) for ALA, CDEPA and DEA were 2.3, 0.8 and 0.8 ng per injection, respectively. The linear fit of analyte to mass response had an R^2 of 0.99. Reproducibility of the sample handling and LC–MS analysis had a RSD of ≤10%. The average recoveries for these analytes in rat plasma were better than 90%. Similar results were obtained with rat urine. Published by Elsevier Science B.V.

Keywords: Alachlor

1. Introduction

Alachlor (2-chloro-*N*-[2,6-diethylphenyl]-*N*-methoxymethyl-acetanilide) (ALA) is an emergent herbicide, millions of pounds of which are used each year in the United States for weed control in corn, potatoes, peanuts and soybeans [1–4]. It has been recognized as a carcinogen in laboratory animals [5–9]. ALA is metabolized by cytochrome P_{450} isoform 3A4 to form at least two major metabolites, 2-chloro-*N*-[2,6-diethylphenyl]acetamide (CDEPA) and 2,6-diethylaniline (DEA) [10–12]. These metabolites have been detected in vitro in human liver microsomes and in urine [11,13]. However, no information is available about the metabolites in vivo after dermal exposures.

Determinations of ALA, CDEPA and DEA are most frequently done using high-performance liquid chromatography (HPLC) in combination with an ultraviolet (UV) detector [14–18] or a radioactivity detector [19–21] after these compounds have been extracted from a complex medium. However, UV detection lacks analyte specificity, and its detection sensitivity is not sufficient. Although radioactivity

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1570-0232/02/$ – see front matter Published by Elsevier Science B.V.
PII: S0378-4347(01)00549-7

detection generally provides sufficient sensitivity for the determinations [22,23], it lacks analyte specificity and requires expensive radiolabeled compounds. In addition, there are other concerns such as radioactive waste treatment, instrument contamination and radiation safety in the use of radiolabeled compounds. Gas chromatography–mass spectrometry (GC–MS), on the other hand, has been used as an analyte-specific detector [24–33]. However, it takes a long time to complete an analysis without sample derivatization. To obtain rapid acquisition, samples have to be derivatized before analysis. Although no information is available about derivatization of alachlor and its metabolites, any derivatization would need to be carried out at high temperature, which can cause artificial oxidation, decomposition products and a reduced level of analyte for detection [34]. Liquid chromatography–mass spectrometry with electrospray ionization (LC–ESI-MS) provides a useful tool for analysis of these analytes [35–37]. This technique does not require sample derivatization and provides excellent specificity and good sensitivity. It also enables researchers to complete an assay for analytes within a short time. Although there are LC–MS related methods [35–37], no one has reported the simultaneous determination of alachlor and its metabolites. The interest in this study concerns cutaneous exposure of chemicals and its biotransformation by skin cytochrome P_{450} (catalyzing ALA to CDEPA and DEA). The objective, therefore, was to develop a simultaneous determination method to generate reproducible qualitative and quantitative data on ALA and these metabolites in rat plasma and urine in preparation for mass-balance studies.

2. Materials and methods

2.1. Reagents

Acetonitrile, methanol (MeOH), acetic acid and water (HPLC grade H_2O) were purchased from Sigma (St. Louis, MO, USA). ALA (CAS: 15972-60-8), CDEPA (CAS: 6967-29-9) and DEA (CAS: 579-66-8) were purchased from ChemService (West Chester, PA, USA). The isotope labeled ALA (ring-$^{13}C_6$, 99%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Instrumentation

2.2.1. LC–MS

A Finnigan dual octapole mass spectrometer (Model LCQ) was coupled through an electrospray ionization (ESI) unit to a ThermoQuest HPLC system. The HPLC system consists of a solvent degas unit with a vacuum pressure of −20″ Hg, a solvent delivery dual pump system (Model P4000), and an autosampler (Model AS3000) with a stream switching valve and a temperature control unit. The instrument control, data acquisition and analyses were performed using Finnigan Xcalibur 1.2 software. The HPLC was fitted with an Xterra MS C_8 column, 2.5-μm particle size, 50 mm×2.1 mm (Waters, Milford, MA, USA).

2.3. Preparation of rat plasma

Normal Sprague–Dawley rats (male and female, 150–280 g) were sacrificed by an overdose of sodium pentobarbital (165 mg/kg). Blood was collected directly from the heart of each rat by open chest cardiac puncture [38]. Blood was placed in a pre-chilled EDTA vacutainer, gently mixed by inversion, and held on ice until centrifuged at 4 °C, 1200 *g* for 30 min. Plasma was carefully transferred to cryovials, and then stored at −40 °C until used.

2.4. Preparation of standard mixture

To obtain suitable standard mixture stock solutions, we prepared 1 μg/μl of ALA, CDEPA, DEA and ^{13}C-labeled internal standard in acetonitrile. Aliquots of each of these solutions were diluted to 10 ng/μl with mobile phase (28% acetonitrile and 72% H_2O, containing 1% acetic acid). Using these solutions, we tuned the instrument for each of the individual compounds. Based on their mass chromatogram response, a mixture with certain ratios of these analytes was prepared and then diluted with mobile phase, by a factor of 2, into different concentrations.

2.5. Solid-phase extraction

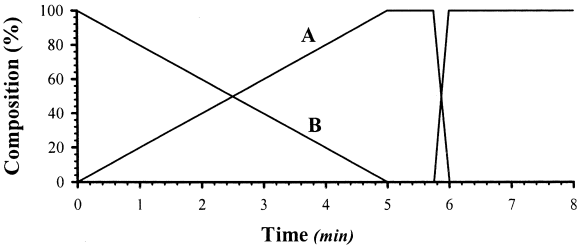
Solid-phase extraction was performed on an extraction vacuum manifold using Waters Oasis HLB

96-well extraction plates (1 cc, 30 mg/well). Before extraction, each well of the plate was conditioned with 1 ml methanol and then equilibrated with 1 ml water. A 50-μl aliquot of standard mixture was mixed with 1 ml rat plasma or 1 ml rat urine by vortexing for ~10 s. The standard mixture consisted of regular ALA, CDEPA, DEA and internal standard (ISTD), ^{13}C-labeled alachlor. Spiked plasma samples then received 20 μl concentrated phosphoric acid while spiked urine samples received 10 μl of 5 *M* HCl. Spiked samples were then vortexed briefly. The 1-ml spiked plasma samples or 1-ml spiked urines samples were then loaded onto the plate with the vacuum released and drawn through at 2.5″ Hg. Subsequently, 1 ml H_2O was added to each well (to wash out impurities such as proteins), followed by 0.5 ml 35% MeOH–2% NH_4OH aqueous solution (to remove other impurities). After this step, the waste tray was replaced with a clean collection plate. The target compounds were recovered by eluting the sorbent twice with a volume equal to half the total desired volume (2×0.5 ml of acetonitrile containing 2% acetic acid). Each eluent was transferred into a 1.5-ml maximum recovery glass vial and evaporated to ~30 μl (never to dryness) with N_2 or under a vacuum. Mobile phase (50 μl of 28% acetonitrile–72% H_2O containing 1% acetic acid) was added and vortexed for ~10 s making the sample ready for LC–MS analysis.

2.6. LC–MS assay

The separation of ALA, CDEPA and DEA was carried out on a ThermoQuest HPLC system fitted with an Xterra MS C_8 column operating at ambient temperature at a constant flow-rate of 0.3 ml/min with the gradient program as shown in Scheme 1.

A 5-μl aliquot of the extracted sample was injected onto the HPLC column. The LC eluents were delivered to the ESI source of the MS, which was operated at 4.5-kV spray voltage with an arbitrary value of 80 for sheath gas flow-rate (nitrogen) at a fixed 100 p.s.i. on its low pressure gauge and 20 arbitrary units of auxiliary gas flow-rate (extra pure helium) at a fixed 40 p.s.i. on its low pressure gauge. The heated capillary was set at 220 °C. The mass spectrometer (LCQ, Finnigan) was operated in the positive selected ion monitoring (SIM) mode during screening of ALA, CDEPA and DEA. Extra pure helium was also used as a buffer gas. The maximum injection time was set at 200 ms and the number of microscans at 3 for all analyses. Analytes were detected by MS using SIM of the individual MH^+ ions in different segments with separate scan events. The details will be described in the Results and discusion section.



Scheme 1. HPLC mobile phase gradient program. Flow rate was set at a constant of 0.3 ml/min at room temperature. (A) Acetonitrile containing 1% acetic acid; (B) 28% acetonitrile–72% H_2O containing 1% acetic acid.

2.7. Quantification of targets

Quantitative analysis was performed using Finnigan Xcalibur 1.2 software to create a processing method and automatically measure the response of the LC–MS system to the compounds in a sample. The response measurement was taken from the area under each peak and is determined by an integration calculation. This measurement, known as the response factor (RF), is calculated as follows:

$$RF = R \cdot [ISTD]/[A_{known}] \quad (1)$$

where *R* is the ratio of peak areas of a known analyte and an internal standard, [ISTD] is the amount of internal standard and $[A_{known}]$ is the known amount of a standard analyte.

The standard calibration curves of ALA, CDEPA and DEA were established to achieve a constant response factor for each compound by the LC–MS system to varying amounts of analyte injected. A constant response factor is indicated by linearly increasing peak area measurements with increasing amounts of analyte injected. The amount of target compound in unknown samples can be determined according to the equation:

$$[A_{\text{unknown}}] = R \cdot [\text{ISTD}]/\text{RF} \quad (2)$$

where $[A_{\text{unknown}}]$ is the amount of a target compound in the unknown sample.

Quantifications of ALA, CDEPA and DEA were done using the ^{13}C-labeled ALA as the ISTD. The responses of each analyte and ISTD were measured at their molecular masses, $[M+H]^+$ m/z and $[M+H]^+ + 6$ m/z by separate events in the same segment of the mass set-up program. The amount was determined for each analyte using a five-point calibration curve generated at each specified mass with six replicate samples.

2.8. Statistical analyses

Statistical analyses of data were made using SigmaStat (Jandel Scientific, San Rafael, CA, USA). Data presented are the means±SD of values compared. The precision of the assay was determined using six replicate samples of rat plasma or urine spiked with a known amount of a target compound in the presence of a fixed amount of ISTD.

3. Results and discussion

This study presents analytical methodology to characterize ALA and its metabolites, CDEPA and DEA, in rat plasma and urine. Methods developed included solid-phase extraction, chromatographic resolution and simultaneous detection of analytes. The results provide the following information.

3.1. LC–ESI-MS chromatogram and spectra of alachlor metabolites

Fig. 1 shows the mass chromatograms and spectra of the standard mixture alone and in spiked rat plasma and urine, respectively. The total ion chromatogram (TIC) in Fig. 1A contains three peaks that were obtained in a spiked rat plasma consisting of DEA (25 ng/ml), CDEPA (25 ng/ml) and ALA (75 ng/ml). The first peak exhibits a Gaussian profile at a mass of 150.2 m/z (see DEA spectrum in Fig. 1D), which is a value consistent with the DEA protonated ion. The second peak shows a Gaussian profile at a mass of 226.2 m/z (see CDEPA spectrum in Fig. 1E), in accord with the CDEPA protonated ion. The third peak consists of a Gaussian profile at a mass 270 m/z (see ALA spectrum in Fig. 1F), a value similar to the ALA positive ion. The retention times for peaks DEA, CDEPA and ALA were determined to be 1.84, 3.11 and 4.12 min, respectively. The retention time differences between the peaks for DEA, CDEPA and ALA were calculated to be 1.27

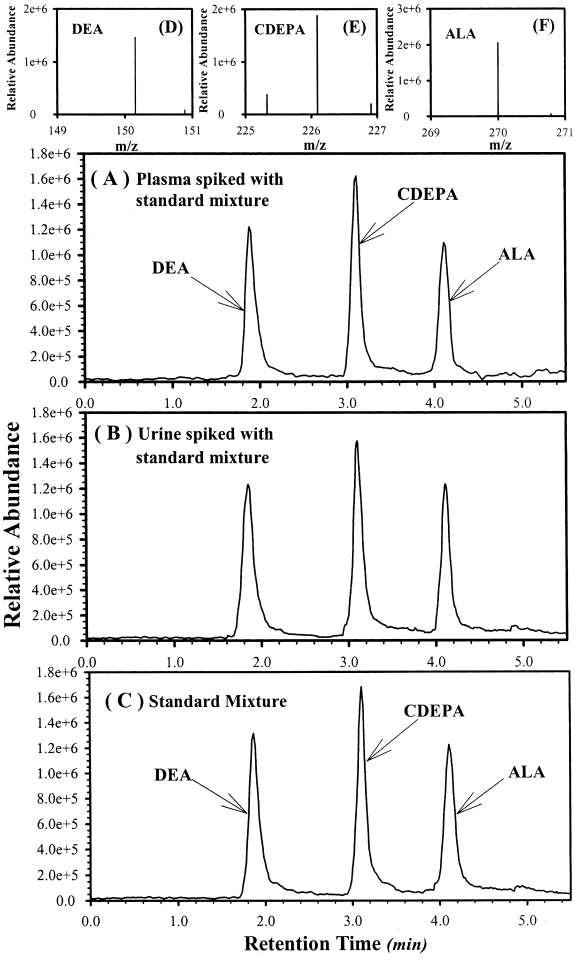


Fig. 1. LC–ESI-MS chromatograms and spectra of alachlor and metabolites. The chromatogram and spectra were obtained from: (A) rat plasma spiked with standard mixture; (B) rat urine spiked with standard mixture; and (C) standard mixture. (D), (E) and (F) are the MS spectra of DEA, CDEPA and ALA, respectively. The concentrations of all samples consisted of 25 ng/ml of DEA, 25 ng/ml of CDEPA and 75 ng/ml of ALA. The HPLC and mass spectrometer settings are described in Section 2.2.1 (LC–MS assay).

and 1.1 min, respectively. The peak widths were measured to be 0.25 min for the first peak, 0.35 min for the second peak and 0.35 min for the third peak. It is evident that the separation of these components is satisfactory. A similar result was obtained for rat urine spiked with standard mixture (Fig. 1B). As shown in Fig. 1B, the mass chromatogram exhibits three peaks with similar mass values and retention times as in Fig. 1A.

To further confirm these peak results from DEA, CDEPA and ALA, we compared the mass chromatograms and spectra obtained from the same standard mixture that was used in spiked plasma and urine under the same conditions. As shown in Fig. 1C, the mass chromatogram and spectra of the standard mixture is the same as those obtained in spiked rat plasma and urine. It is evident that the three peaks obtained in spiked rat plasma and urine resulted from DEA, CDEPA and ALA.

3.2. Quantitative assay and intra-assay validation

Quantification of total analyte during sample preparation was accomplished using the ^{13}C-labeled ALA as the ISTD. The responses of each analyte and ISTD were measured at their molecular masses by separate segments and events with their own tuning method in the mass detector set-up program. This measurement was taken from the area under each peak by an integration calculation. Fig. 2 shows such a determination in which the LC–MS chromatograms of ALA and its metabolites are presented in different segments. DEA was monitored in the retention time range of 0–2.75 min at SIM mode 150.2±1 m/z, and CDEPA was monitored in the retention time range of 2.75–3.75 min at 226.2±1 m/z. ALA and ^{13}C-ALA were traced in the retention time range of 3.75–5.5 min at 270±1 and 276±1 m/z in the same segment with different scan event, respectively. The total amount in the spiked sample was determined for each analyte using a five-point calibration curve generated at each specified mass with six replicate samples in the presence of a fixed amount of ^{13}C-ALA. The results of these determinations are summarized in Table 1. The relative standard deviation (RSD) was within the range of ±10%. It is evident that the intra-assay validation precision is satisfactory.

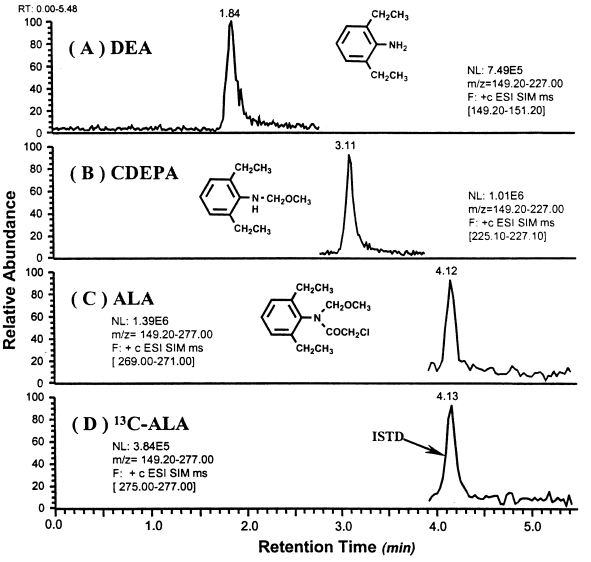


Fig. 2. LC–MS chromatograms of alachlor and metabolites obtained in spiked urine presented in different segments. These species were detected in different ranges of retention time and mass. (A) DEA, 0–2.75 min and 149.2–151.2 m/z; (B) CDEPA, 2.75–3.75 min and 225.2–227.2 m/z; (C) ALA, 3.75–5.50 min and 269–271 m/z; and (D) ^{13}C-ALA, 3.75–5.5 min and 275–277 m/z.

3.3. Linearity of mass response curves of samples spiked with standards

Linearity of ALA, CDEPA and DEA to mass response was verified by measuring the correlation coefficient (R^2) for each calibration curve. A total of six replicate samples of each of the five different concentrations were analyzed using the ratio of the peak areas of analytes and ISTD. If a method is reliable, the ratio of an analyte with ISTD should increase linearly with the amount of the analyte injected, and the plot of the ratio versus the amount of analyte should give a straight line with a constant slope and a high correlation coefficient. As shown in Fig. 3, the ratio increased as a function of analyte concentration, and the plots of standard mixture and standard mixture spiked samples gave a straight line with a satisfactory correlation coefficient for ALA, CDEPA and DEA in the standard mixture and the spiked samples (Fig. 3A, B and C), respectively. The RSD values for these analytes varied within the range of ±10%, which is widely accepted in bio-

Table 1
Summary of the assay validation data from spiked rat plasma and urine

Spiked plasma					
[ALA] ng/ml	37.5	75	150	300	600
Mean ($n=6$, ng/ml)	40.29	78.84	149.28	281.45	609.96
SD	2.61	5.02	10.93	18.66	37.51
%RSD	6.47	6.36	7.32	6.63	6.18
[CDEPA] ng/ml	12.5	25	50	100	200
Mean ($n=6$, ng/ml)	11.63	24.11	49.18	99.14	199.14
SD	0.80	1.65	2.68	4.94	10.40
%RSD	6.92	6.85	5.44	4.98	5.22
[DEA] ng/ml	12.5	25	50	100	200
Mean ($n=6$, ng/ml)	13.07	23.96	50.64	99.68	199.97
SD	1.05	2.15	3.37	5.42	10.42
%RSD	8.03	8.97	6.65	5.44	5.21
Spiked urine					
[ALA] ng/ml	37.5	75	150	300	600
Mean ($n=6$, ng/ml)	33.82	72.14	147.28	288.11	601.37
SD	2.70	6.10	14.29	18.55	36.74
%RSD	7.97	8.45	9.70	6.44	6.11
[CDEPA] ng/ml	12.5	25	50	100	200
Mean ($n=6$, ng/ml)	14.49	25.33	47.25	93.91	195.70
SD	1.35	2.22	2.94	9.37	9.96
%RSD	9.32	8.77	6.21	9.98	5.09
[DEA] ng/ml	12.5	25	50	100	200
Mean ($n=6$, ng/ml)	12.77	22.67	51.81	101.81	198.55
SD	0.98	2.26	3.31	5.37	10.35
%RSD	7.68	9.96	6.38	5.27	5.21

All data are the calculated amounts of analyte based on the peak area ratio to ISTD.

analytical work [39,40]. This indicates that the method is reliable and may be applied to ongoing in vivo bio-transformation studies.

3.4. Recovery of ALA and its metabolites in rat plasma and urine

To further examine the reliability of the developed method for these compounds in rat plasma and urine, an experiment was conducted using low and high concentrations of the standard mixtures to spike plasma and urine and calculate the recoveries of ALA, CDEPA and DEA. Samples of 1 ml plasma or 1 ml urine (each spiked with 50 μl of one standard mixture) were extracted using Oasis HLB extraction plate (1 cc, 30 mg/well, 96 wells) and analyzed in six replicates. The standard deviations from each set of samples are small (Table 2). Although the value of 92% for ALA in high level spiked plasma is slightly low and 108, 109 and 107% for others at low level seem high, the values are within an acceptable ±range. It indicates that the method is reliable.

3.5. Sensitivity

The sensitivity of a method is very important for trace analysis and mainly depends upon instrument

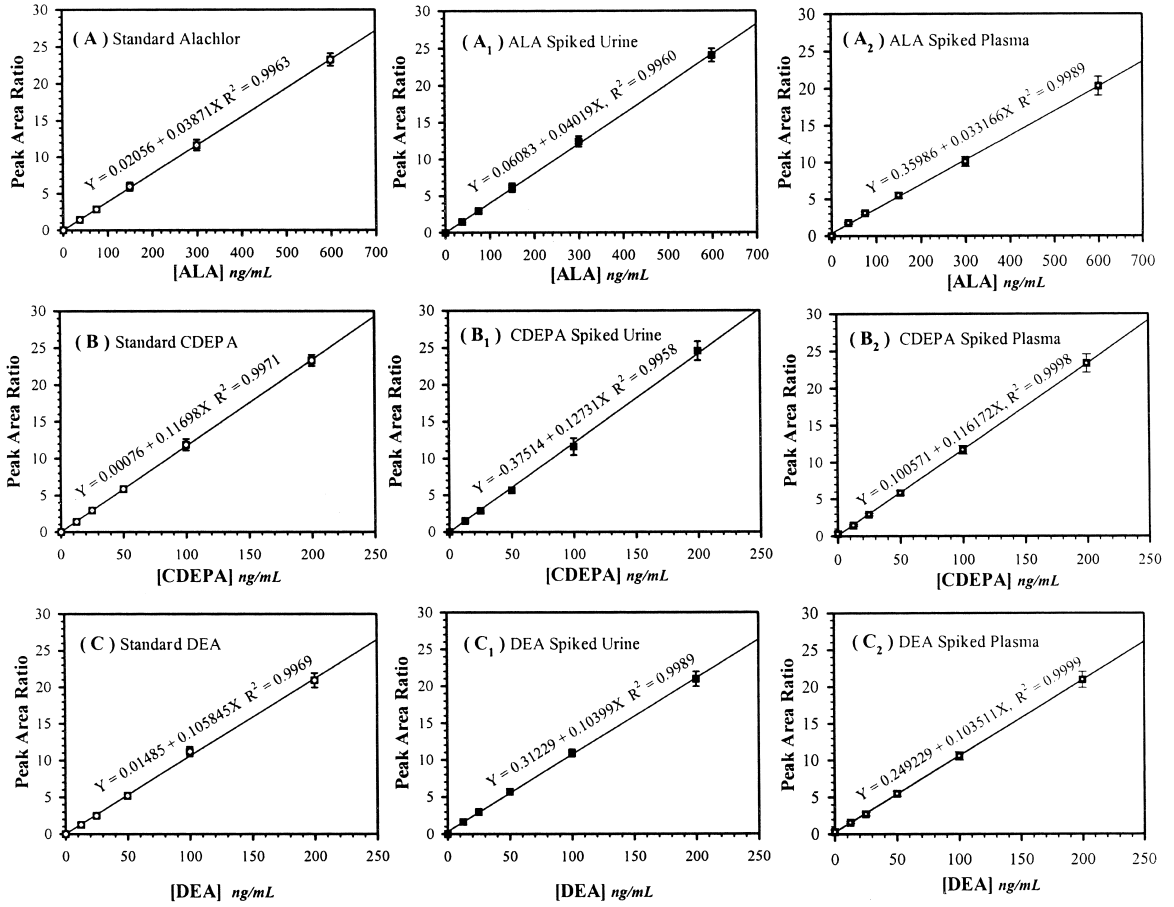


Fig. 3. Comparison of the curves of alachlor and its metabolites. (A)–(C) standard mixture; (A_1)–(C_1) rat plasma spiked with standard mixture; and (A_2)–(C_2) rat urine spiked with standard mixture. The amount of individual analyte is indicated in each plot.

sensitivity. The limit of quantification (LOQ) was obtained by determining the lowest amount of each analyte using a standard calibration curve and Xcalibur 1.2 software. The acceptable variability of the measured amount for each analyte was set at ±10%. The LOQ for ALA, CDEPA and DEA were

Table 2
% Recoveries of alachlor and its metabolites in rat plasma and urine

Compound spiking level	ALA		CDEPA		DEA	
	75 ng/ml	600 ng/ml	25 ng/ml	200 ng/ml	25 ng/ml	200 ng/ml
StdM ($n=6$)	2.86±0.25	23.24±1.48	2.94±0.25	23.32±0.96	2.50±0.23	20.94±0.98
StdM/PL ($n=6$)	3.08±0.20	21.30±1.26	2.92±0.20	23.38±1.22	2.70±0.242	20.97±1.10
% Recovery	108	92	99	100	109	100
StdM/U ($n=6$)	2.96±0.25	24.03±1.48	2.85±0.22	24.54±1.25	2.67±0.28	20.96±1.09
% Recovery	104	103	97	105	107	100

StdM, standard mixture; StdM/PL, plasma spiked with standard mixture; StdM/U, rat urine spiked with standard mixture. All data are presented with the ratio of peak area to ISTD.

found to be 2.3, 0.8 and 0.8 ng per injection, respectively.

The methodology developed in this study has the important advantages of producing a sensitive and interference-free analysis. First, ESI-MS produces a spectrum in which nearly all of the ion current is in the pseudo-molecular ion $[M+H]^+$, which gives improved sensitivity and specificity. Second, sample preparation procedures generate a clean analyte that is protein contaminant-free and the analysis time is shorter than many other methods. The method for the simultaneous determination of ALA, CDEPA and DEA in rat plasma and urine was validated successfully in the analytical concentration range of 12.5–200 ng/ml for CDEPA and DEA, and 37.5–600 ng/ml for ALA. The intra-assay validation results show small standard deviations (Table 1). The mass peak area response curves for the set of calibration standards and spiked samples were linear with an R^2 of 0.99 (Fig. 3). The recoveries for all analytes analyzed were acceptable. The results illustrate that the method is reliable and can be applied to many ALA metabolite bio-transformation studies in vivo. Specifically, the method will be employed in an ongoing ALA dermal penetration and bio-transformation study and qualitative and quantitative characterization of ALA and its metabolites.

4. Nomenclature

ALA	2-chloro-*N*-[2,6-diethylphenyl]-*N*-methoxymethyl-acetanilide
CDEPA	2-chloro-*N*-[2,6-diethylphenyl]-acetamide
DEA	2,6-diethylaniline
ESI	electrospray ionization
LC	liquid chromatography
MS	mass spectrometry

Acknowledgements

We are grateful to Dr Sidney Soderholm for corrections and useful suggestions.

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