Analytical Biochemistry **299,** 227–234 (2001)
doi:10.1006/abio.2001.5423, available online at http://www.idealibrary.com on IDEAL®

Rapid Determination of Soluble Epoxide Hydrolase Inhibitors in Rat Hepatic Microsomes by High-Performance Liquid Chromatography with Electrospray Tandem Mass Spectrometry

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Received July 6, 2001; published online November 15, 2001

A rapid and reliable electrospray tandem mass spectrometric method for soluble epoxide hydrolase (sEH) inhibitors in rat hepatic microsomes is described. Four synthesized sEH inhibitors were extracted from rat hepatic microsomes with ethyl acetate and were determined by HPLC using positive ion electrospray tandem mass spectrometry within 7 min. The relationship between signal intensity and concentration of sEH inhibitors was linear over the concentration range of 2.0 to 500 ng/mL per 5-μL injection with the use of a noncoeluting internal standard with a similar chemical structure. The intraassay precision was less than 12.4% relative standard deviation and accuracy ranged from −7.0 to 11.3% deviation from the theoretical values with five duplicate assays. The recovery of sEH inhibitors from rat hepatic microsomes, fortified at levels of 50, 100, and 250 ng/mL, averaged 74.2–107.7% with a RSD of 2.1–7.6%. This method was successfully applied to the quantification of residual sEH inhibitors in rat hepatic microsomes without interference.

Key Words: **soluble epoxide hydrolase; quantification; inhibitor; rat hepatic microsomes; liquid chromatography; electrospray ionization; tandem mass spectrometry.**

Epoxide hydrolases (EH, EC 3.3.2.3)[2] are enzymes that add water to epoxides (1). These enzymes are widely distributed throughout the animal and plant kingdoms and not only metabolize epoxides of drugs and xenobiotics, but also catalyze the hydration of endogenous compounds. In mammals, there are two major forms of EH, the microsomal EH and soluble EH (sEH), which detoxify mutagenic, carcinogenic, and xenobiotic epoxides (2, 3). The soluble or cytosolic enzyme also is involved in the metabolism of epoxides of arachidonic acid (4) (*cis*-epoxyeicosatrienoic acids) and linoleic acid (leukotoxin and isoleukotoxin) (5). Epoxides of arachidonic acid are well known as blood pressure regulators (6), while the diols of linoleate epoxides have been implicated in inflammatory disorders, such as acute respiratory distress syndrome (5), and may be endogenous regulators of vascular permeability and inflammation (7). Therefore, the modulation of endogenous lipid epoxides by use of sEH inhibitors appears to have therapeutic benefits in both hypertensive and inflammatory conditions. We have already reported the synthesis of potent and stable urea, amide, and carbamate inhibitors of sEH and applied them to *in vitro* and *in vivo* models (8–10) which function equally well on murine, rat, and human enzyme orthologs. In this paper, we focus on four potent disubstituted urea inhibitors as shown in Fig. 1. As a first step to estimating the availability of sEH inhibitors as *in vivo* pharmacological agents, a specific, accurate, and rapid quantification method is required to assess *in vitro* metabolic stability. The absence of chromophores in

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[2] Abbreviations used: EH, epoxide hydrolase; sEH, soluble epoxide hydrolase; GC-MS, gas chromatography–mass spectrometry; LC-MS, liquid chromatography with mass spectrometry; DCU, 1,3-dicyclohexyl-urea; CAU, 1-adamantan-1-yl-3-cyclohexyl-urea; CDeU, 1-cyclohexyl-3-decyl-urea; CDU, 1-cyclohexyl-3-dodecyl-urea; CTU, 1-cyclohexyl-3-tetradecyl-urea; APcI, atmospheric *z*-spray pressure chemical ionization; ESI, electrospray ionization; MRM, multiple reaction monitoring; CID, collision-induced dissociation; RSD, relative standard deviation; QC, quality control; LOQ, limit of quantification; TIC, total ion chromatogram; LOD, limit of detection.

0003-2697/01 $35.00

the structure of the most potent urea inhibitors rules out their detection using optical spectroscopic methods, such as UV and fluorescence spectroscopy. Gas chromatography–mass spectrometry (GC-MS) remains one of the most extensively employed methods. However, many of these sEH inhibitors are not amenable to GC-MS detection because of low volatility. This drawback can be overcome by derivatization (11); however, the additional sample treatment is time-consuming and can lead to interference. Liquid chromatography with mass spectrometry (LC-MS) provides a potential tool not only for our target compounds in drug metabolism studies, but also for urea pesticides (12, 13). In particular, LC-MS and LC-MS-MS are proven techniques for the rapid and sensitive determination of drugs and metabolites (14–16) in the pharmaceutical field.

This paper describes a rapid, sensitive, and selective ESI-LC-MS-MS method for the simultaneous determination of sEH inhibitors using a short reversed-phase LC column. The method is applied as an *in vitro* metabolism assay of rat hepatic microsome samples.

EXPERIMENTAL

Materials. 1,3-Dicyclohexyl-urea (DCU) was obtained from Aldrich (Milwaukee, WI). 1-Adamantan-1-yl-3-cyclohexyl-urea (CAU), 1-cyclohexyl-3-decyl-urea (CDeU), 1-cyclohexyl-3-dodecyl-urea (CDU), and 1-cyclohexyl-3-tetradecyl-urea (CTU) (internal standard) were synthesized in our laboratory as previously reported (17, 18). These products were purified by recrystallization and characterized structurally by ^{1}H and/or ^{13}C NMR and infrared and mass spectroscopy.

HPLC-grade methanol, acetonitrile, and ethyl acetate; reagent-grade sodium phosphate monobasic monohydrate; anhydrous sodium phosphate dibasic; and formic acid were purchased from Fisher Scientific (Pittsburgh, PA). Glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide phosphate sodium salt (NADP), and D-glucose-6-phosphate monosodium salt were obtained from Sigma (St. Louis, MO), and anhydrous magnesium chloride was purchased from Aldrich and used in a NADPH-generating system. Water (>17.9 MΩ) used was purified using a NANO pure II system (Barnstead, Newton, MA).

Sample preparation. Stock solutions of sEH inhibitors were prepared by dissolving 5–10 mg of each compound in 20–40 mL of methanol. Standard solutions were stored at 4°C in the dark. One microgram per milliliter standard solutions were used for the MS study. The concentration range in the calibration studies varied from 2 to 500 ng/mL. A stock solution of CTU for internal standard was prepared in methanol and diluted to a concentration of 500 ng/mL with methanol.

Instrumentation. The LC-MS-MS system consisted of a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an atmospheric pressure ionization source [atmospheric *z*-spray pressure chemical ionization (APcI) or electrospray ionization (ESI) interface]. The HPLC system consisted of a Waters Model 2790 separations module (Waters Corporation, Milford, MA) equipped with Waters Model 2487 dual λ absorbance detector. The mass spectrometer was coupled to the outlet of HPLC column or syringe pump for optimization with PEEK tubing (50 × 0.128 mm i.d.). Data were manipulated with MassLynx software (Ver. 3.5).

LC-MS-MS conditions. Chromatographic separation was performed using XTerraMS C_{18} column (3.5 μm, 30 × 2.1 mm i.d.) (Waters Corporation) at ambient temperature. Solvents A and B were 0.1% formic acid in distilled water and acetonitrile containing 0.1% formic acid, respectively. Solvents were degassed by vacuum. Mobile phases were delivered at a flow rate of 0.3 mL/min with a linear gradient from 40% B to 100% B over 0–5 min and held isocratic for 8 min with 100% B. Typically, 5μL of standard or the extracted microsome samples were injected onto the column.

The ESI was performed in positive ion mode with a capillary voltage of 1.0 kV. The source and the desolvation temperature were set at 100 and 300°C, respectively. Cone gas (N_2) and desolvation gas (N_2) were maintained at flow rates of 140 and 640 L/h, respectively. The optimum cone voltages were 40 V for DCU, 90 V for CAU, 90 V for CDeU, 80 V for CDU, and 100 V for CTU (internal standard), respectively. The APcI was performed in the positive mode. Ionization was carried at a corona current of 0.1 μA and at a source and probe temperature of 125 and 450°C, respectively. Cone gas (N_2) and desolvation gas (N_2) were maintained at flow rates of 75 and 220 L/h, respectively. The cone voltage was set at 100 V for DCU, CDU, and CDeU and 90 V for CAU, respectively. Mass spectra of the precursor ions were obtained by direct injection and scanning over the range of 50 to 350 m/z at 3 s/scan. Data were acquired in the MCA and continuum modes. Quantitative analysis was performed in the multiple reaction monitoring (MRM) mode with a dwell time of 800 ms. For (CID), argon (99.9999%) was used as a collision gas at a pressure of 2.5 mTorr.

Microsomal incubations. Male Fischer 344 rats were used for isolation of liver microsomes by standard procedure (19). Protein concentration was determined by the Bradford method (20). Eight-hundred ninety microliters of liver microsomal preparations in 100 mM sodium phosphate buffer at pH 7.4, corresponding to 0.2 mg of microsomal protein in a total volume of 1 mL, was preincubated for 5 min in open glass tubes immersed in a shaking bath at a constant temperature of

37°C. After incubation, 10 μL of 100 μM substrate solutions was added to liver microsomes, and then the reaction was initiated by the addition of 100 μL of the NADPH-generating system, which consisted of 2 mM NADP, 57 mM glucose 6-phosphate, 3.5 units/mL glucose-6-phosphate dehydrogenase, and 50 mM magnesium chloride dissolved in 100 mM sodium phosphate buffer at pH 7.4. Each incubation mixture (1 mL total volume) was incubated in a shaking water bath kept at 37°C for 5, 10, 20, 30, 40, or 60 min. A control was prepared by the addition of 1 mL of ethyl acetate after adding the NADPH-generating system. Reactions were terminated by the addition of 1 mL of ethyl acetate. Two-hundred microliters of 500 ng/mL internal standard was added to the samples. The samples were then vortexed and centrifuged at 6000 rpm (4000*g*) for 5 min. The aqueous phases were extracted twice with ethyl acetate, and extracts were combined and dried under nitrogen. The residue was reconstituted in 1 mL of methanol. Aliquots (5 μL) were injected onto the LC-MS-MS system.

Assay validation. The assay for simultaneous determination of the four sEH inhibitors was validated over the range from 5 to 250 ng/mL. The calibration curves were calculated by weighted least square regression ($1/x^2$) analysis of the ratios of the analyte to the internal standard peak areas against the corresponding analyte concentrations. The precision and the accuracy of the assay were expressed as the relative standard deviations (RSD) and the percentage of deviation from the theoretical value, respectively. In the intraassay, five replicates of quality control (QC) samples were analyzed at each concentration level. All samples were analyzed on the same day and their calculated sample concentrations were determined from calibration curves prepared in series during the same run. The limit of quantification (LOQ) of the assay was defined as the lowest concentration on the calibration curve, with acceptable precision (<20%) and accuracy (<20%). The recovery was assessed using the microsome QC samples. The rat hepatic microsomes were spiked with 50, 100, and 250 ng/mL standard of each inhibitor. The internal standard was added to rat hepatic microsomes before the extraction procedure described above. The recovery was determined by comparing the mean chromatographic peak responses for each analyte to the peak responses of equivalent amounts of standard.

RESULTS AND DISCUSSION

Chemical structures of DCU, CDeU, CDU, CAU, and CTU (internal standard) are shown in Fig. 1. All analytes were efficiently ionized in the positive mode due to the presence of the urea nitrogens in the structure. The sensitivity of ESI and APcI was compared in the positive mode. In the case of ESI, the protonated molecule (precursor ion) $[M + H]^+$ was the most abundant ion and the $[M + H + ACN]^+$ adduct ion was also observed with approximately half the abundance of $[M + H]^+$ in the Q1 scan spectra of all analytes except for DCU. On the other hand, the precursor ion $[M + H]^+$ and $[M + H + ACN]^+$ adduct ion were also observed in APcI Q1 scan spectra; however, the abundances of all analytes were lower than found in the ESI Q1 scan spectra. Consequently, the precursor ions of all analytes were set at each protonated molecule $[M + H]^+$. For MS-MS experiments, the precursor ions of each analyte were as follows: DCU *m/z* 225; CDeU *m/z* 283; CDU *m/z* 311; CAU *m/z* 277; CTU 339. The ESI tandem mass product ion spectra resulting from CID of each molecular ion are depicted in Fig. 2. As expected, similarities in the fragment patterns (N-C bond cleavage) were observed for all analytes. In the case of APcI, the fragment patterns were also similar to ESI. The most abundant product ions were *m/z* 83 (DCU), *m/z* 135 (CAU), *m/z* 201 (CDeU), *m/z* 229 (CDU), and *m/z* 257 (CTU; internal standard), respectively. Therefore, these product ions were used for the MRM scan. Figure 3 shows the effect of collision voltage on the sensitivity of each analyte (CTU not shown). The collision voltage affects the transmission and fragmentation of analyte ions. In general, higher collision voltage will enhance fragmentation, reducing the abundance of large frag-

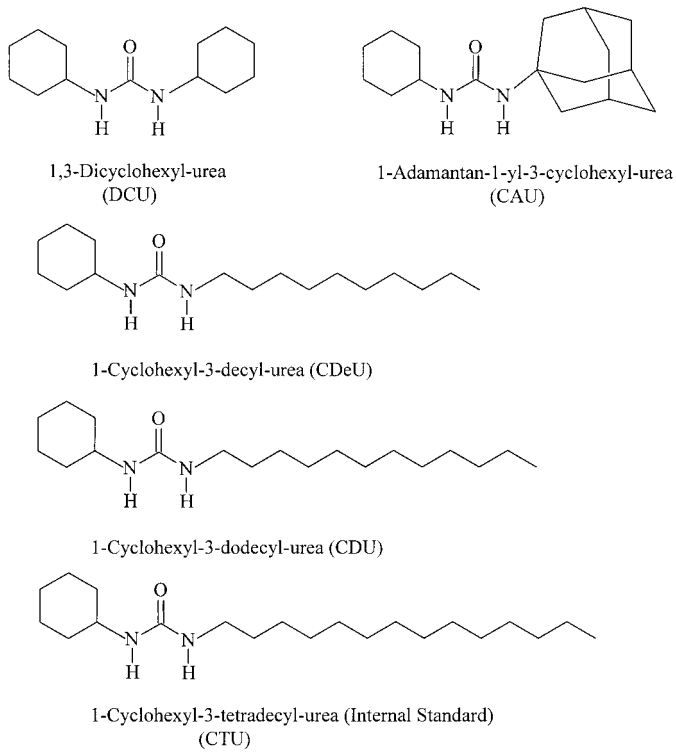

FIG. 1. Structures of sEH inhibitors.

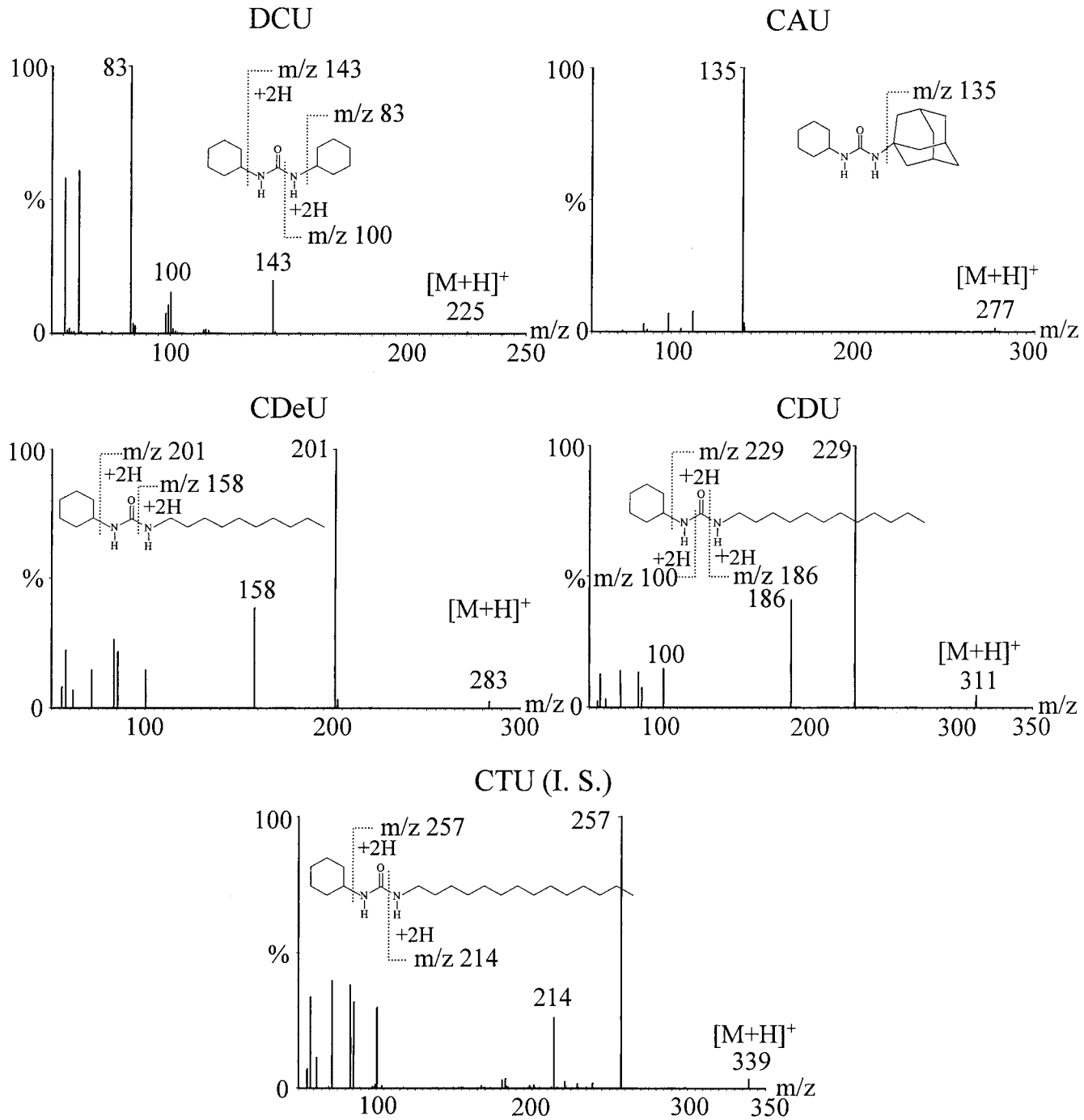

FIG. 2. ESI tandem mass spectra of sEH inhibitors.

ments. However, the high abundance of *m*/*z* 135 derived from adamantane was constant in the high collision voltage range. These analytes were hydrophobic compounds. While the analysis of nonpolar compounds usually prefers the APcI probe to the ESI probe, the abundances of all the analyzed ureas under ESI were significantly higher than under APcI. Therefore, ESI was used to set up the MRM mode for the quantification.

To optimize the MS-MS conditions, the changes in the precursor ion and the product ions were carefully observed under the various collision voltages in the range from 10 to 32 eV. As shown in Table 1, at low collision voltages below 15 eV there was very little

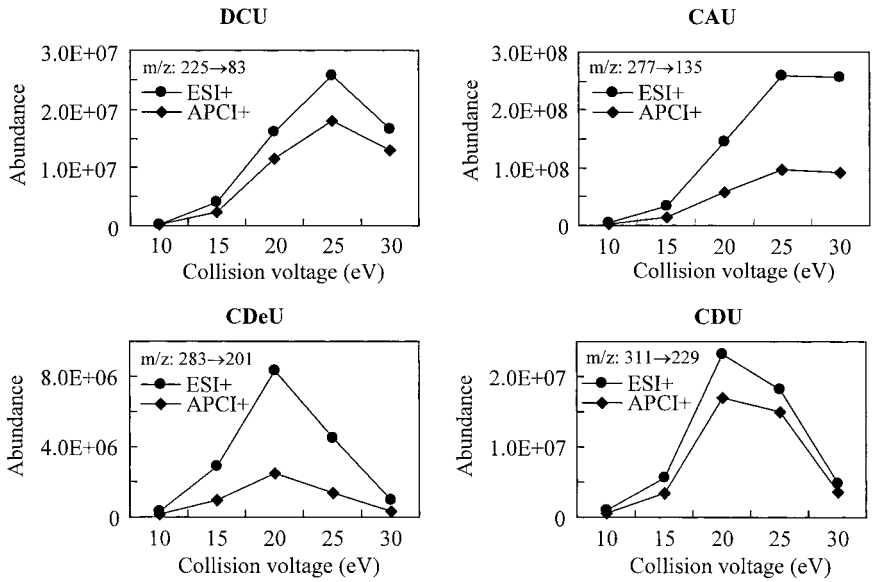

FIG. 3. Effect of collision voltage on product ions.

generation of the characteristic product ions. The intensities of the precursor ions were decreased with an increase in the collision voltage. On the other hand, the intensities of the product ions were relatively increased, but their intensities were decreased at the high collision voltage. Superficially the collision voltage that generated the most abundant product ion should be chosen as the optimum ionization conditions. However, such conditions could result in a high background. Therefore, considering sensitivity, background, and the characterization of analyte, the optimum collision voltages of all the analytes were set at 30 eV for DCU and CTU (internal standard), 32 eV for CAU, and 27 eV for CDeU and CDU, respectively. Separation conditions were investigated to shorten the analytical time using a short column (30 × 2.1 mm i.d.).

A total ion chromatogram (TIC) and MRM profiles of sEH inhibitor standards under ESI LC-MS-MS conditions are shown in Fig. 4. One nanogram of DCU,

TABLE 1

Effect of Collision Voltage on the Relative Abundance of sEH Inhibitors

Compound	Ions *m/z*	Collision voltage (eV):	Relative abundance (%) 10	15	20	25	27	30	32
DCU	225 $[M + H]^+$		100	100	64.4	10.3	2.6	0.6	0.2
	143		0	12.8	47.4	82.6	41.7	19.6	11.7
	100		4.2	30.7	100	84.7	39.0	15.1	0
	83		0.1	1.3	23.8	100	100	100	100
CAU	277 $[M + H]^+$		100	100	77.3	15.0	12.5	2.1	1.2
	135		2.3	8.8	100	100	100	100	100
	100		0.3	1.5	10.9	3.9	3.2	0	0
CDeU	283 $[M + H]^+$		100	100	100	3.5	2.4	4.5	3.9
	201		0.6	10.5	79.2	100	100	100	100
	158		1.0	22.5	88.7	81.7	38.6	47.8	47.9
	100		0.6	8.4	65.8	43.2	14.6	48.2	38.5
CDU	311 $[M + H]^+$		100	100	100	17.6	4.4	1.2	1.6
	229		0.4	4.9	37.4	100	100	100	100
	186		0.7	14.3	47.9	58.8	40.7	37.4	37.7
	100		0.7	10.5	41.9	49.3	14.7	20.2	50.7
CTU (internal standard)	339 $[M + H]^+$		100	100	100	31.9	12.4	3.3	2.2
	257		1.0	3.0	26.0	100	100	100	100
	214		0.4	4.6	31.2	48.3	34.0	26.2	27.5

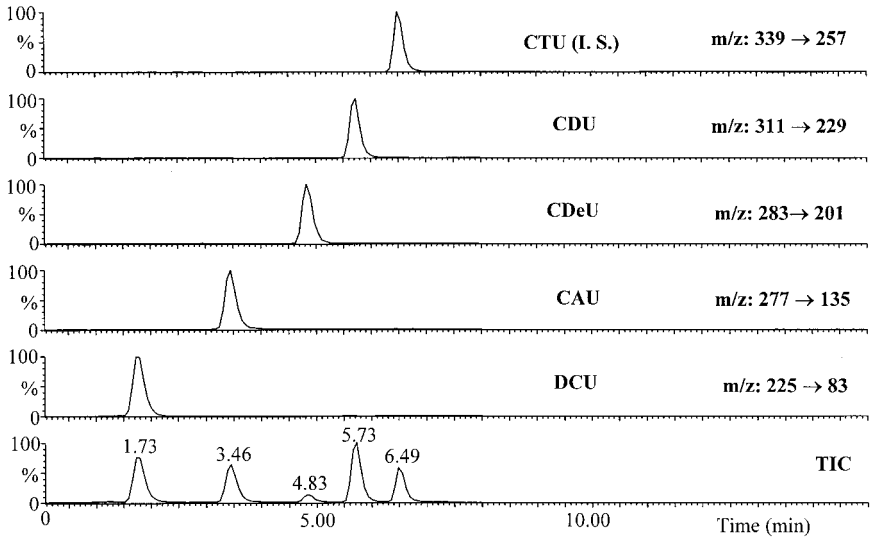

FIG. 4. Total ion chromatogram and MRM profiles of sEH inhibitors standard under ESI LC-MS-MS conditions.

CDeU, CDU, and CTU and 0.2 ng of CAU were injected onto the LC-MS-MS system. All peaks of sEH inhibitors were completely separated within 7 min. The elution from the column was in order of DCU (1.7 min), CAU (3.5 min), CDeU (4.8 min), CDU (5.7 min), and CTU (6.5 min). In general, the flow rate of the semimicrocolumn (inner diameter of 2.1 or 2.0 mm) is set at 0.2 mL/min for optimal performance. We then assessed whether the optimal separation and sensitivity were required for this assay. When the analytes were separated at the flow rate of 0.2 mL/min, it took 13 min to achieve full separation of the analyte and no improvement of sensitivity was observed. Consequently, we confirmed that a flow rate of 0.3 mL/min was reasonable for the separation of our analytes using a semimicrocolumn (30 × 2.1 mm i.d.).

Calibration curves were constructed from the peak ratios of sEH inhibitors to CTU calculated from MRM profiles. Good linearities were observed for all the analytes between the concentration of sEH inhibitor and the response corresponding to the peak area ratios in the concentration range from 2 to 500 ng/mL ($r > 0.99$). The limits of detection (LODs) were calculated using a signal-to-noise ratio of 3. LODs of these analytes were as follows: 0.2 ng/mL for CAU, 0.5 ng/mL for DCU and CDU, and 1 ng/mL for CDeU. The intraassay precision and accuracy for the assay were determined by analyzing five replicates at QC concentration of 5.0, 50, and 250 ng/mL for all analytes. All samples were analyzed on the same day and their back-calculated concentrations were determined from simultaneously generated calibration curves. The intraassay precision and accuracy data are shown in Table 2. The RSDs of DCU, CDU, CDeU, and CAU at 5.0 ng/mL were 11.2, 9.6, 12.4, and 8.8%, respectively. The percentages of deviations (accuracy) of all analytes at 5.0 ng/mL were between −3.8 and 11.3%. Precision and accuracy are the most important criteria in the assessment of an analytical method (21). The LOQ is defined as the lowest concentration that can be determined with an acceptable accuracy of ±20% and a precision less than 20%. Therefore, the LOQs of all analytes were determined as 5.0 ng/mL. These results indicated that the present analytical method has satisfactory precision and accuracy.

The recoveries of sEH inhibitors from rat hepatic microsomes are summarized in Table 3. Following spiking at a concentration of 50, 100, and 250 ng/mL the analyte was extracted from the rat hepatic microsomes by liquid–liquid partition. Although a few peaks

TABLE 2

Intraassay Precision and Accuracy for Quantification of sEH Inhibitors

Compound	Theoretical value (ng/mL)	Observed value (ng/mL)	RSD (%)	Accuracy (%)
DUC	5.0	5.2	11.2	4.8
	50	48	6.5	−3.5
	250	239	4.7	−4.4
CDU	5.0	4.8	9.6	−3.8
	50	47	4.4	−5.4
	250	266	3.2	6.2
CDeU	5.0	5.1	12.4	2.5
	50	49	5.2	−1.4
	250	233	3.5	−7.0
CAU	5.0	5.6	8.8	11.3
	50	52	10.1	3.3
	250	236	5.3	−5.4

Note. $n = 5$.

TABLE 3

Recovery of sEH Inhibitors from Spiked Rat Hepatic Microsomes

Compound	Spiked (ng/mL)	Recovery (±RSD) %
DCU	50	105.1 ± 2.1
	100	104.0 ± 4.7
	250	99.5 ± 7.6
CDU	50	101.9 ± 5.9
	100	107.7 ± 4.6
	250	102.5 ± 5.1
CDeU	50	74.2 ± 5.0
	100	88.1 ± 4.8
	250	95.2 ± 7.3
CAU	50	91.2 ± 2.9
	100	97.7 ± 6.3
	250	96.0 ± 7.9

Note. $n = 5$.

derived from the rat microsomes were found in the TIC, each of the analyte peaks was clearly observed in MRM profiles without interfering peaks. The recovery obtained for DCU, CDU, CDeU, and CAU at 50 ng/mL was 105.1, 101.9, 74.2, and 91.2%, respectively. The recovery of the analytes except for CDeU at 50 and 100 ng/mL was determined between 91.2 and 107.7%. We have developed a series of sEH inhibitors with K_i's approaching 1 nM. Several of these compounds demonstrate activity in rodents *in vivo* in lowering blood pressure and reducing inflammation. As a step toward optimizing these compounds as tools for cardiovascular research, an appreciation of their metabolic stability is critical. A concentration of 1 μM was selected for the microsomal inhibition studies as being a pharmacologically realistic concentration.

The ESI LC-MS-MS quantitative method was applied to the determination of the residual primary compound in rat hepatic microsomes. Figure 5 shows the percentage of the residual compound in the rat hepatic microsomes after incubation. The residual amount of DCU was still 66% after 60 min of incubation. On the other hand, metabolism of CDeU and CDU was very fast in microsomes because the alkyl chain in their structures could be easily metabolized by P450 (22, 23). The more rapid metabolism of CAU compared to DCU was surprising since CAU was anticipated to be more sterically hindered. However, the very high sensitivity of the assay based on the 135 amu adamantyl fragment suggests that this substituent could be useful for studies of *in vivo* pharmacokinetics. Oxidation at the ω and ω-1 position is anticipated to contribute to the rapid metabolism of CDeU and CDU. There was no detectable loss of any compound in the absence of the NADPH-generating system.

There is no metabolism of any of the compounds in the absence of NADPH. Figure 5A demonstrates that CDeU and CDU are rapidly and completely metabolized in microsomes with NADPH. DCU and CAU are metabolized more slowly, and the metabolism appears to plateau before the parent compound is completely destroyed. We tested several obvious hypotheses to explain this observation. NADPH was not found to be rate limiting during the course of the reaction out to 1 h, and the activity of the microsomal system on CDU was found to be the same when measured at various times out to 1 h. Thus the explanation for the plateau effect was not provided either by a loss of NADPH or by decomposition of P450 during the reaction. Other hypotheses to explain the plateau effect are in process.

In conclusion, a rapid, sensitive, and specific ESI LC-MS-MS method for the quantification of sEH inhibitors in rat hepatic microsomes was developed. The present method provided sufficient sensitivity with reasonable precision and accuracy for all analytes and was successfully applied to *in vitro* metabolism assay of sEH inhibitors without interference. Although we demonstrated the utility of the ESI LC-MS-MS method for an *in vitro* metabolism assay in the present study, the general procedure can also be used for pharmacokinetic studies of sEH inhibitors and the identification and/or determination of sEH inhibitor metabolites in biological fluids.

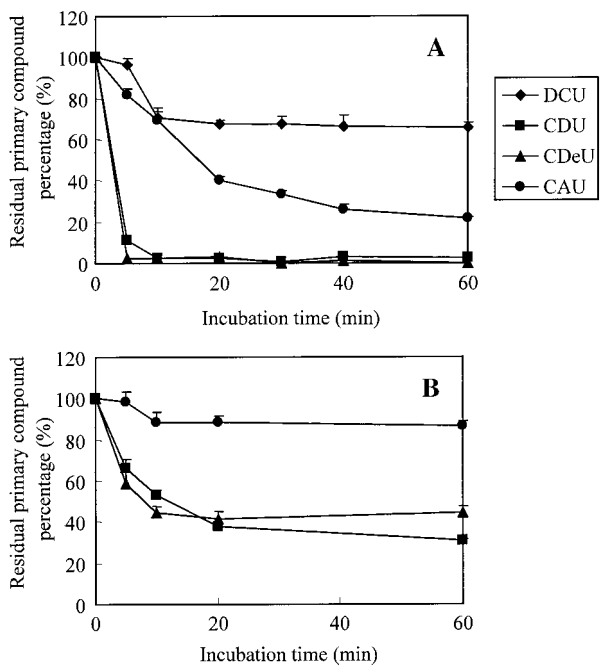

FIG. 5. Residual primary compound percentage in rat hepatic microsomes. The initial concentration of each compound was 1 μM and the internal standard was added after the termination of the reaction at a concentration of 100 ng/mL. In A microsomes were at a concentration of 0.2 mg protein while in B they were at a concentration of 0.02 mg protein. Data are expressed as means ± SD of triplicate experiments.

ACKNOWLEDGMENTS

This work was supported in part by NIEHS Grant R37 ES02710, NIEHS Superfund Basic Research Program Grant P42 ES04699, and NIEHS Center for Environmental Health Sciences Grant P30 ES05705. We thank J. W. Newman and C. Morisseau for helpful discussions.

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