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Determination of norepinephrine in small volume plasma samples by stable-isotope dilution gas chromatography–tandem mass spectrometry with negative ion chemical ionization

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Abstract

A stable-isotope based gas chromatography–tandem mass spectrometry–negative ion chemical ionization method was developed for the determination of norepinephrine (NE) levels in small volumes (25–100 μl) of plasma. NE was stabilized in plasma by the addition of semicarbazide and spiked with deuterium-labeled norepinephrine internal standard. The analytes were isolated from the plasma by solid-phase extraction using phenylboronic acid columns and derivatized using pentafluoropropionic anhydride. The derivatized analytes were chromatographed on a capillary column and detected by tandem mass spectrometry with negative ion chemical ionization. Unparalleled sensitivity and selectivity were obtained using this detection scheme, allowing the unambiguous analysis of trace levels of NE in small-volume plasma samples. Linear standard curves were obtained for NE over a mass range from 1 to 200 pg per sample. The method had a limit of quantitation of 10 pg NE/ml plasma when using a 100-μl sample aliquot (1 pg/sample). Accuracy for the analysis of plasma samples spiked with 10 to 200 pg NE/ml typically ranged from 100±10%, with RSD values of less than 10%. The methodology was applied to determine the effect of clonidine on plasma NE levels in conscious spontaneously hypertensive rats. Administration of clonidine (30 μg/kg) produced an ~80% reduction in plasma NE accompanied by a 30% reduction in heart and mean arterial pressure that persisted >90 min after drug administration. The ability to take multiple samples from individual rats allowed the time course for the effect of clonidine to be mapped out using only one group of animals.

Keywords: Norepinephrine

1. Introduction

Norepinephrine (NE, see Fig. 1A), a member of the endogenous catecholamine family, plays a central biological role as a hormone and neurotransmitter in mammals. Abnormal secretion and/or metabolism of NE is important in the diagnosis of a number of disease states such as pheochromocytoma and neuroblastoma [1,2], in evaluating the etiology of neuroendocrinology disorders [3], as well as providing an index of overall activity of the sympathetic nervous system [4]. Additionally, sympathetic nervous system activity, as evidenced by elevated circulating plasma concentrations of NE plays an important role

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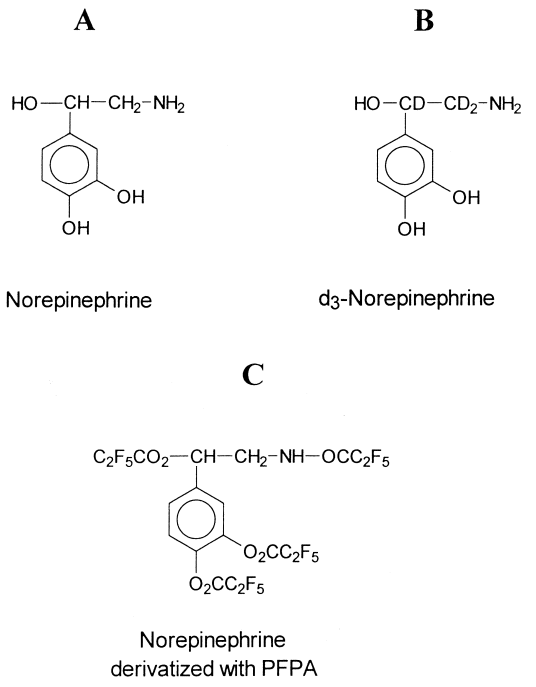

Fig. 1. Structure of: (A) norepinephrine (NE), (B) α,β,β-d_3-norepinephrine (d_3-NE) and (C) NE derivatized with pentafluorpropionic anhydride.

in cardiac hypertrophy and failure [5–7]. The spontaneously hypertensive rat (SHR) is an experimental model of hypertension and cardiac hypertrophy associated with elevated sympathetic nervous system activity. Monitoring plasma levels of NE over time allows an assessment of the sympathetic activity.

Due to the low endogenous levels of circulating NE and the complex nature of the biological matrices, highly sensitive and selective methodologies are required for the analysis of NE. In general, extensive sample preparation is required prior to the analysis of NE from biological matrices. Solid-phase extraction (SPE) using alumina [8,9], phenylboronic acid [10,11] or ion-exchange [12] has been applied extensively, although liquid–liquid extraction [13,14] and ultrafiltration methods [15] have also been employed. A variety of analytical methods have been utilized for the analysis of NE in plasma, urine and cerebrospinal fluids including radioenzymatic reactions [16], high-performance liquid chromatography (HPLC) with fluorescence [12,13,17] and electrochemical [9,15,18] detection and gas chromatography with mass spectrometry (GC–MS) [19–21]. Both the HPLC–fluorescence and the GC–MS methodologies require the derivatization of NE prior to analysis. HPLC with both fluorescence and electrochemical detection have reported NE detection limits in the 1 pg/ml range [22,23] and have been applied for the analysis of NE in small (100 μl) sample volumes [15]. In general, however, the chromatographic profiles obtained from both approaches result in extraneous matrix peaks and sloping baselines that complicate the analysis of NE in plasma samples. GC–MS detection schemes based on the use of electron-capturing derivatives using negative ion chemical ionization (NICI) have provided the capability for low level detection of NE [20]. Although the MS detection scheme provides a high degree of selectivity, in our hands extraneous matrix components are still observed, hindering the determination of NE at low levels and/or in small plasma volumes.

We report on the development of the first stable-isotope based gas chromatography–tandem mass spectrometry–negative ion chemical ionization (GC–MS–MS–NICI) method for the analysis of NE in small volumes (10 to 100 μl) of rat and dog plasma. NE was isolated from plasma using a semi-automated SPE on phenylboronic acid columns and derivatized with pentafluoropropionic anhydride. NE was quantitated using a stable-isotope based GC–MS–MS–NICI method. The selectivity of the tandem mass spectrometry approach results in unparalleled selectivity for the analysis of NE levels from a plasma matrix. The electron capturing derivative of NE allows the use of an NICI approach for high sensitivity. The GC–MS–MS–NICI method was found to be rugged, giving excellent accuracy and precision for the analysis of NE in small volumes of plasma.

The utility of the methodology for monitoring NE as a marker of sympathetic nervous system activity was validated in SHR that were administered a centrally acting sympathioinhibitory agent, clonidine. High sensitivity for the analysis of NE plasma levels was required due to the need to obtain multiple samples from the same rat over a short time period. NE levels were determined prior to and following administration of clonidine by taking multiple small-volume samples from individual animals.

2. Experimental

2.1. Chemicals and reagents

L-NE, DL-7-^{3}H-norepinephrine ([^{3}H]NE, 185 GBq/mmol, 10 μg/ml) and α,β,β-d_3-norepinephrine (d_3-NE, 99% labeled, see Fig. 1B) were obtained from Lancaster Synthesis (Windham, NH, USA), New England Nuclear (Wilmington, DE, USA) and Isotec (Miamisburg, OH, USA), respectively. Glacial acetic acid (reagent grade), ethyl acetate (reagent grade), methylene chloride (HPLC grade), methanol (HPLC grade), acetonitrile (HPLC grade), ammonium hydroxide (28–30%), ammonium sulfate and 0.1 *M* HCl were from J.T. Baker (Philipsburg, NJ, USA). Ethyl acetate was distilled over calcium hydride prior to use and stored between uses in a desiccator. Distilled–deionized water was obtained from a Barnstead NANOpure II system (Dubuque, IA, USA) and semicarbazide hydrochloride was obtained from Sigma (St. Louis, MO, USA). Pentafluoropropionic anhydride (PFPA) was obtained from Regis Technologies (Morton Grove, IL, USA). Heptafluorobutyric acid (HFBA) was from Aldrich (Milwaukee, WI, USA). Rat and dog plasma containing EDTA as an anticoagulant were obtained from Pel-Freez Biologicals (Rogers, AR, USA). Whole blood was obtained from Sprague–Dawley rats at Procter and Gamble's Miami Valley Labs. (Cincinnati, OH, USA) according to the required animal care activities.

2.2. NE standards

A 1 mg/ml NE stock solution was prepared by dissolving NE in 1 *M* acetic acid. Appropriate dilutions of the 1 mg/ml stock solution were made in 1 *M* acetic acid to give additional NE stock solutions of 100 pg/ml and 1 ng/ml. Similarly, a 1 mg/ml d_3-NE stock solution was prepared in 1 *M* acetic acid and appropriate dilutions of this solution were performed in 1 *M* acetic acid to yield a 29 ng/ml d_3-NE stock solution. The stock solutions for NE and d_3-NE were stored at −70°C and were stable for over 3 months. NE working standards from 1 to 200 pg were prepared by aliquoting an appropriate volume of either the 100 pg/ml or 1 ng/ml NE stock solutions into 0.7-ml autosampler vials already containing 25 μl of the 29 ng/ml d_3-NE stock solution. The solutions were then vortex-mixed and taken to dryness under nitrogen using a Reacti-Vap III (Pierce, Rockford, IL, USA). The residue for each standard was then derivatized and processed as described below.

2.3. NE plasma and whole blood stability

Whole blood or whole blood containing 0.1 *M* semicarbazide hydrochloride were spiked with [^{3}H]NE. A sample of the spiked whole blood was centrifuged to yield plasma either immediately after spiking or after remaining 2 h at room temperature. The plasma obtained after centrifugation was immediately treated with two volumes of acetonitrile, vortexed, centrifuged and the supernatant analyzed by reversed-phase HPLC with on-line radiochemical detection (HPLC–RAD) as described below. Similarly, plasma or plasma containing 0.1 *M* semicarbazide hydrochloride were spiked with [^{3}H]NE. A portion of each sample was immediately treated with acetonitrile and processed as above, while a second portion of each sample was allowed to remain at room temperature for 2 h before the addition of the acetonitrile. Again, the supernatant from these samples was analyzed by HPLC–RAD. HPLC–RAD analysis was done using a Waters (Milford, MA, USA) Symmetry ODS column (15 cm×3.9 mm) with a mobile phase of water modified with HFBA (1.3 ml HFBA/l) at a flow-rate of 1.0 ml/min. The effluent from the HPLC column was mixed with scintillation cocktail (Flo-Scint II, Packard, Meriden, CT, USA) at a ratio of 1:2 before entering the 0.5-ml flow cell of the Flo-One Beta radiochemical detector (Packard).

2.4. Preparation of spiked plasma validation samples

An aliquot of 1 *M* aqueous semicarbazide solution was added to either blank rat or dog plasma at a ratio of 1:10. Aliquots of the blank dog plasma were then spiked to contain NE at the 10, 25, 50, 100, 200, 500 or 2000 pg/ml level, while the blank rat plasma was spiked to contain NE at the 200 pg/ml level. Then aliquots, 100 μl for dog (n=5) and 5, 10, 25 and 100 μl for rat (n=3), of either blank or spiked plasma

samples, were added to individual 2-ml polypropylene cryovials already containing 100 μl of 0.5 *M* phosphate buffer (pH 7.4) and 25 μl of the 29 ng/ml d_3-NE stock solution. The tubes were then capped and the contents briefly mixed by vortexing. The spiked dog or rat samples were then prepared for analysis by SPE as described below.

2.5. Animal sample processing

Whole blood samples (0.2 ml) were spiked with 20 μl of 1 *M* aqueous semicarbazide solution, gently mixed by inversion and then the plasma separated by centrifugation. The plasma samples were stored frozen at −70°C until analysis. On the analysis day, samples were thawed, mixed by inversion and a portion of the samples (25 to 100 μl) were added to individual 2-ml polypropylene vials already containing 100 μl of 0.5 *M* phosphate buffer (pH 7.4) and 25 μl of the 29 ng/ml d_3-NE stock solution. The tubes were then capped and the contents briefly mixed by vortexing. The samples were then prepared for analysis by SPE as described below.

2.6. SPE sample preparation

SPE was done using phenylboronic acid columns (1 ml, 100 mg) obtained from Varian (Harbor City, CA, USA) with an Applied Separations (Allentown, PA, USA) Speed Wiz instrument. The columns were conditioned consecutively with 2 ml of 1 *M* acetic acid–methanol (6:94, v/v), 1 ml distilled–deionized water, 1 ml of 0.1 *M* HCl, 1 ml of 0.3% ammonium hydroxide and 2 ml of 0.01 *M* ammonium sulfate solution (pH 8.3) using a flow-rate of 10 ml/min. Aliquots (25 to 100 μl) of blank plasma, spiked plasma control or unknown animal samples were applied to the conditioned phenylboronic acid columns, at a flow-rate of 1 ml/min, by hand. The columns were then washed consecutively, at a flow-rate of 10 ml/min, with 1 ml of 0.01 *M* ammonium sulfate (pH 8.3), 1 ml of distilled–deionized water and 1 ml of methanol. Finally, the cartridges were eluted with 6 ml of 1 *M* acetic acid–methanol (6:94, v/v) at a flow-rate of 4 ml/min into 100×16 mm Pyrex test tubes. The collected eluent was then taken to dryness on a TurboVap evaporator (Zymark, Hopkington, MA, USA) using nitrogen (138 kPa) at 35°C. The samples were then reconstituted with 0.3 ml of 1 *M* acetic acid–methanol (6:94, v/v), transferred to 0.7-ml conical autosampler vials and taken to dryness under vacuum using a Savant (Farmingdale, NY, USA) Speed-Vac.

2.7. Derivatization

The dried residues for the standards, control samples or unknown samples were derivatized by adding 100 μl of a PFPA–ethyl acetate (1:2, v/v) derivatizing solution to each of the autosampler vials. The contents of the vials were then mixed by vortexing and left covered at room temperature for 30 min. The derivatization solution was then evaporated to dryness under nitrogen using a Reacti-Vap III (Pierce). The residues in the autosampler vials were reconstituted with 50 μl of methylene chloride, capped with PTFE-lined caps and vortex-mixed.

2.8. Absolute recovery of NE from the sample preparation procedure

The absolute recovery of NE from the SPE, drying and derivatization steps were determined by spiking blank rat plasma, treated with semicarbazide, with [^{3}H]NE at the 690 pg/ml level. Aliquots (100 μl) of the spiked rat plasma ($n=7$) were then carried through the SPE, drying and derivatization steps described above. Aliquots of the SPE eluents, the derivatized samples and the final reconstituted sample were then analyzed by liquid scintillation counting on a Packard Model 2000CA Liquid Scintillation Analyzer (Packard). Absolute recovery was determined for each step relative to the direct scintillation analysis of the spiked plasma sample.

2.9. GC–MS–MS–NICI

The GC–MS–MS equipment consisted of a Hewlett-Packard (Palo Alto, CA, USA) 5890 Series II plus gas chromatograph, a Finnigan MAT (San Jose, CA, USA) A200S autosampler and a Finnigan MAT TSQ 700 mass spectrometer equipped with the TSQ 7000 EI-CI ion source. Samples were injected (2 μl) in the splitless mode (1.0 min) into a Hewlett-Packard single-gooseneck liner, packed with a small amount of silanized glass wool, maintained at 150°C.

The PFPA derivatized NE and d_3-NE were chromatographed on a Restek (Bellefonte, PA, USA) Rtx-5 capillary column (30 m×0.25 mm, 0.5 μm film) using an initial column temperature of 110°C for 1 min, followed by a linear temperature ramp to 250°C at a rate of 15°C/min. The column was inserted into the EI-CI source through a transfer line maintained at 250°C. The source and the quadrupoles were maintained at 220 and 80°C, respectively. The column pressure was initially 207 kPa for 1 min, followed by a ramp (276 kPa/min) to 69 kPa with a hold at 69 kPa for 1.5 min and a final ramp (14 kPa/min) to 166 kPa. Ionization was achieved by electron attachment in a methane chemical ionization plasma at a nominal pressure of 9000 mTorr (1 Torr=133.322 Pa). The collision gas was argon (1.5 mTorr) and the collision energy was 10 eV. The PFPA derivatized NE and d_3-NE were detected using selected-reaction monitoring (SRM) schemes involving transitions from *m/z* 442→322 and *m/z* 445→325, respectively.

2.10. Quantification

Electronically integrated peak areas were obtained for the standards and the peak-area ratios (PARs) were computed by dividing the peak area for NE by that of d_3-NE for each standard. The response factor (RF) for each standard was calculated by dividing the mass of NE contained in the calibration standard by its corresponding PAR. The response factors for all the standard levels were averaged to obtain the mean response factor (MRF). The MRF was used to determine the concentrations of NE in unknown and spiked plasma samples by multiplying the PAR obtained for each sample by the MRF and dividing by the volume of the sample used for the analysis.

2.11. Animal study

2.11.1. Surgery

All procedures were approved by the institution's committee on animal care and usage. Male SHRs weighing between 270 and 330 g were anesthetized with isofluorane and air. A catheter (PE10 heat-fused to PE50) was inserted into the abdominal aorta via the femoral artery and a second catheter (0.25×0.04 in. Micro-Renathane) was placed in the superior vena cava via the jugular vein (1 in.=2.54 cm). Catheters were flushed with 100 units/ml heparin to maintain patency and sealed with stainless steel pins. The free ends of the catheters were routed subcutaneously and exteriorized through a small incision in the midscapular region, and secured to the underlying musculature by a ligature of 4.0 silk. They were then threaded through a plastic or stainless steel button used to tether the rat in a conscious state during the experimental procedure. All incisions were closed with 9 mm wound clips. Rats were placed under a heat lamp until they awakened from the anesthetic. Recovered rats were allowed free access to food and water and were allowed 2 to 4 days to recover prior to being used in an experiment.

2.11.2. Experimental protocol

On the experimental day, the arterial catheter of each animal was connected to a rotating swivel mechanism (Instech Labs., Plymouth Meeting, PA, USA). The swivel was connected to a P10EZ Isolated Pressure Transducer (Spectramed, Oxnard, CA, USA), connected to a Gould TA5000 physiograph (Gould, Valleyview, OH, USA) and a computer-based data acquisition and analysis system (Gould/PO-NE-MAH, Valleyview, OH, USA) for continuous measurement of mean arterial pressure and heart rate. Cardiovascular parameters were digitized at 250 Hz and values averaged over 6 s time intervals. Each rat was then placed in its home cage where it could move freely, and was allowed to acclimatize for 30 min. The last 10 min of acclimation time were used to obtain baseline cardiovascular measurements prior to compound administration. A blood sample (250 μl) for NE analysis was taken at approximately this time. Then the animals received an intravenous (i.v.) injection of saline. A second blood sample was obtained 15 min later, followed immediately by i.v. injection of either the α_2-adrenoceptor agonist, clonidine, or saline. Additional blood samples for NE determination were taken 30, 60 and 90 min after administration of clonidine or saline. Semicarbazide was added to the blood samples at a final concentration of 0.1 *M* to slow the possible degradation of NE by monoamine oxidases [24].

3. Results

3.1. Stability of NE in plasma and whole blood

Catecholamines are relatively unstable chemical structures, being easily oxidized, even in the absence of a metabolically active matrix such as plasma. However, the literature contains contradictory data in regards to the plasma stability of NE. Some studies claim NE is stable in plasma [25–27], while other studies indicate NE is rapidly degraded by the plasma matrix [28,29]. We found [^{3}H]NE to be unstable in rat blood and plasma at room temperature, degrading in some studies to give a polar degradant that elutes in the void volume during the HPLC–RAD analysis (Fig. 2A) and degrading in other studies to products that did not elute from the HPLC column (data not shown). While degradation was always observed, the extent and rate of the degradation varied from lot-to-lot of rat plasma or whole blood. In some cases, [^{3}H]NE was completely degraded within 1 h at ambient temperature, while in other cases less than 20% of the [^{3}H]NE had degraded after 1 h. The degradation of catecholamines by a plasma amine oxidase enzyme that is inhibitable by semicarbazide has been reported [24]. The addition of semicarbazide to whole blood or plasma was found to stabilize [^{3}H]NE (Fig. 2B) in whole blood or plasma from the same lot that had previously degraded the [^{3}H]NE (Fig. 2A). The variability in stability of [^{3}H]NE from lot-to-lot of rat whole blood and plasma found in our study, and the contradictory results in the literature, could potentially be explained by varying levels of the amine oxidase as a result of the blood sampling technique.

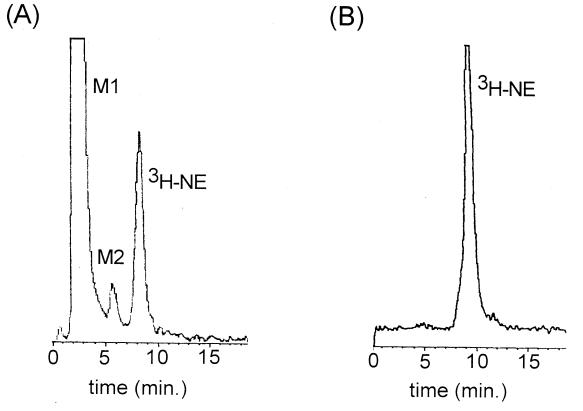

Fig. 2. HPLC with on-line radiochemical detection profiles obtained for blank plasma spiked with ^{3}H-norepinephrine ([^{3}H]NE) and containing: (A) no amine oxidase inhibitor and (B) semicarbazide as an amine oxidase inhibitor. M1 and M2 denote peaks corresponding to enzymatic degradation products of [^{3}H]NE.

3.2. Absolute recovery of NE from sample preparation procedure

The absolute recovery of NE through the SPE, drying and derivatization steps was determined using plasma spiked with [^{3}H]NE. The total recovery of [^{3}H]NE in the SPE effluent was found to be 58±2.3%; following solvent removal and derivatization the total recovery was 37±8.8% and in the final reconstituted sample the total recovery was 19±2.6%. Significant, but relatively reproducible, losses of NE were encountered at each step of the sample preparation procedure. The loss of material during the derivatization and dry down steps could have been due to adsorption onto surfaces or incomplete dissolution in the small volume of methylene chloride. The use of a stable-isotope internal standard is critical for correcting for sample losses.

3.3. GC–MS–NICI and GC–MS–MS–NICI of derivatized norepinephrine

The NICI mass spectrum obtained for derivatized NE using methane as the reagent gas is shown in Fig. 3. There are several major peaks observed in the spectrum, including the molecular radical anion at m/z 753 produced by electron attachment and a prominent peak at m/z 442 corresponding to the loss of both $C_2F_5CO_2H$ and $C_2F_5CO^{\cdot}$ from the molecular anion.

The product ion spectrum of the molecular anion (m/z 753) yields the perfluoropropionate anion (m/z 163) as the only significant peak under the ion activation conditions available with argon at a variety of collision energies (data not shown). For SRM, the 753→163 transition is relatively non-selective since the m/z 163 fragment is common to all compounds derivatized by PFPA. The analysis of plasma samples using the m/z 753→163 SRM

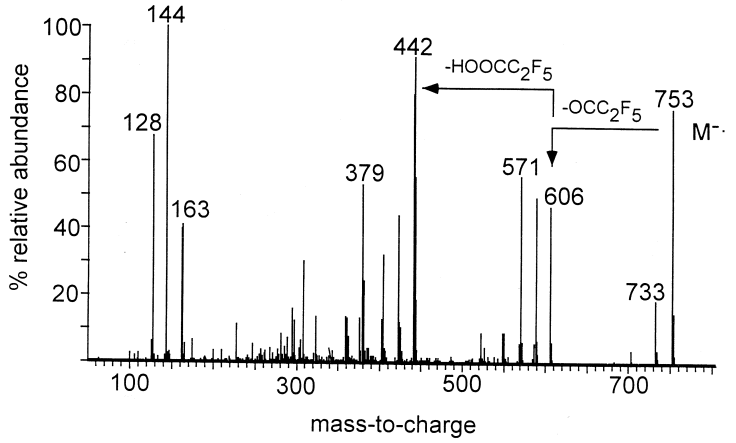

Fig. 3. Full scan negative ion chemical ionization mass spectrum of NE derivatized with pentafluoropropionic anhydride.

scheme produced a chromatographic profile containing a number of extraneous matrix peaks eluting close to NE (data not shown). In fact, the chromatographic profile obtained from this SRM scheme was essentially identical to that obtained by selected-ion monitoring using the *m*/*z* 753 ion (data not shown).

The product ion spectrum of *m*/*z* 442 yields several peaks that are more structurally characteristic of the derivatized NE (Fig. 4). The most abundant of these is a fragment ion at *m*/*z* 322 corresponding to a further loss of C_2F_5H. The SRM scheme, *m*/*z* 442→322, greatly increases the specificity of the method for quantitative applications and was therefore selected for further method development. Corresponding fragmentation patterns are observed with d_3-NE in both the single stage and tandem mass

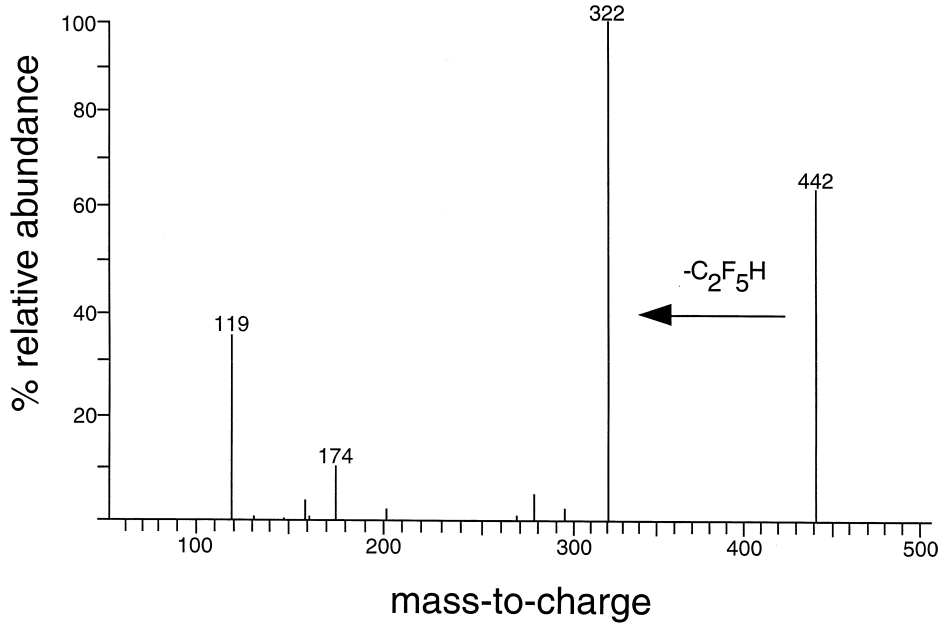

Fig. 4. Negative product ion mass spectrum of *m*/*z* 442 which is a fragment ion of the quadruply derivatized NE generated in the ion source.

spectra, except that m/z values are shifted by three units higher due to the incorporation of the deuterium atoms (data not shown). Therefore, d_3-NE was monitored using an SRM scheme of m/z 445→325.

3.4. GC–MS–MS–NICI of standards and spiked plasma samples

Calibration standards for the NE plasma analysis were prepared using neat solutions due to the presence of unknown and variable levels of endogenous NE that were found to be present in different lots of blank plasma. Additionally, the level of NE in blank plasma pools was found to change over time further complicating the use of the actual matrix for preparing standards. Standards covered a NE range from 1 to 200 pg and resulted in linear calibration curves with correlation coefficients of 0.999 or better. The chromatographic profile obtained for a 1 pg standard (40 fg NE on-column), containing 725 pg d_3-NE (29 pg on-column), is shown in Fig. 5. NE eluted from the GC column with good peak shape and a S/N ratio of greater than 5 was always obtained for the 1 pg standard.

Fig. 6A and B show the chromatograms obtained for 0.025 ml samples of blank rat plasma spiked with d_3-NE (29 ng/ml) and blank rat plasma spiked with both NE (500 pg/ml) and d_3-NE (29 ng/ml), respectively. The chromatographic profiles obtained for the blank plasma samples were relatively free of extraneous peaks due to endogenous materials, high-

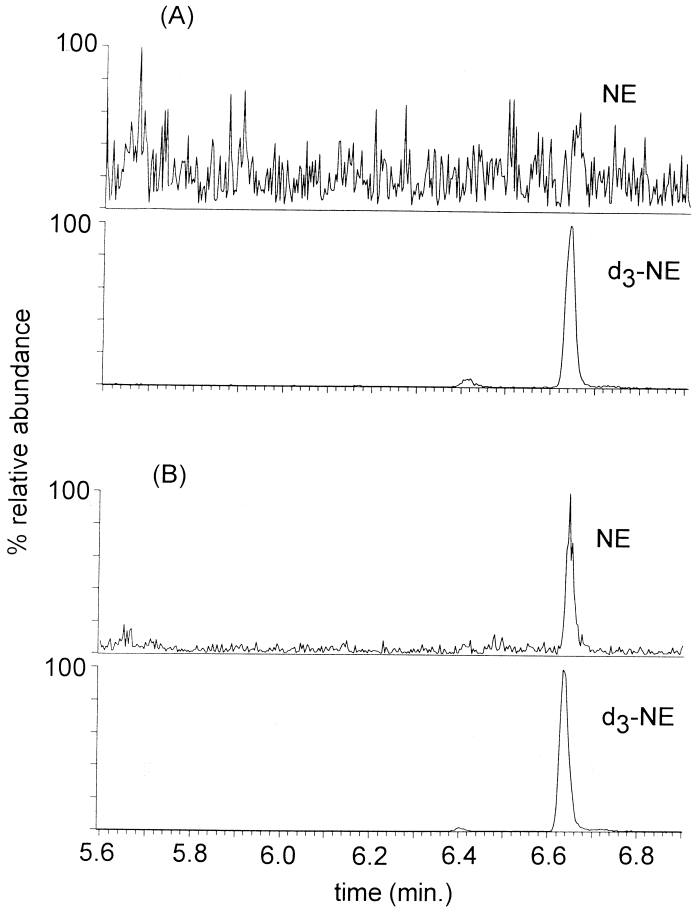

Fig. 5. Selected-reaction monitoring chromatograms for a 2-μl injection of neat solutions (50 μl) spiked with (A) d_3-NE (725 pg) and (B) d_3-NE (725 pg) and NE (1 pg). The NE trace in (B) represents ~40 fg of NE on-column.

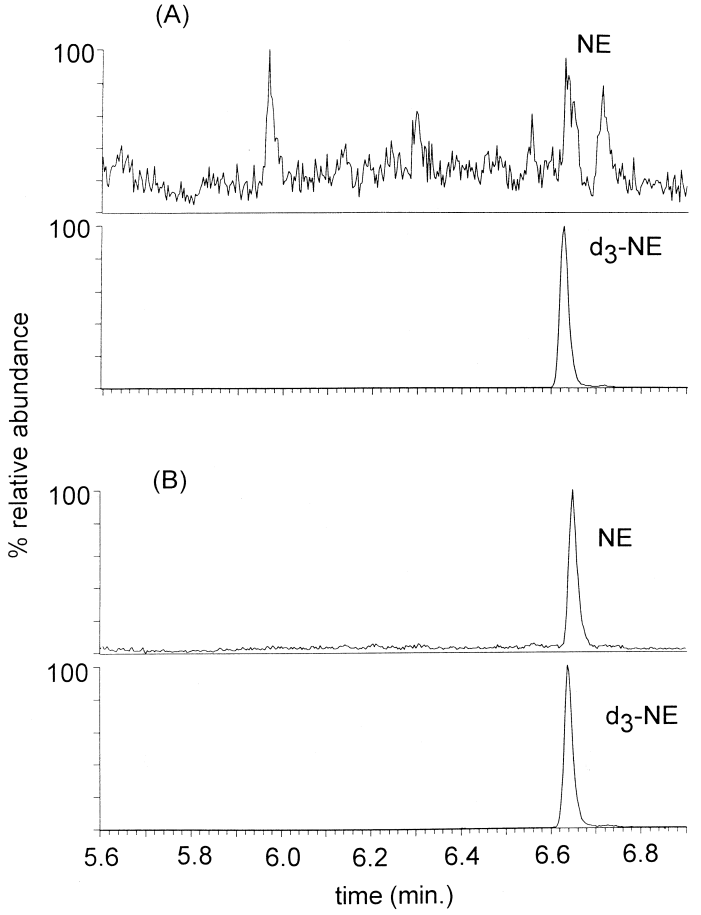

Fig. 6. Selected-reaction monitoring chromatograms for a 25-μl rat plasma sample spiked with (A) d_3-NE and (B) d_3-NE and 500 pg/ml NE.

lighting the selectivity of the SRM scheme used for the analysis. A small peak was observed in the blank rat plasma for endogenous NE. The spiked plasma sample contained peaks corresponding to the added NE and d_3-NE.

3.5. Accuracy and precision of NE plasma analysis – dog and rat

The analysis of small volumes (100 μl) of dog plasma spiked with various levels of NE ($n=5$) are shown in Table 1 for two separate analysis days. In general, the accuracy was within ±10% of target, with RSD values of less than 8.5%, for spiked NE levels ranging from 10 to 2000 pg/ml. Dog plasma was selected for this portion of the accuracy and precision study because various lots of blank dog plasma were found to contain consistently low and reproducible levels of NE. The lots of dog plasma

Table 1
Accuracy and precision of norepinephrine (NE) determination in spiked dog plasma samples (100 μl sample volume; $n=5$)

Spiked NE concentration (pg/ml)	% Recovery (RSD, %)	
	Day 1	Day 2
10	94 (6.8)	103 (8.2)
25	95 (8.4)	96 (7.5)
50	90 (4.4)	91 (4.8)
100	90 (5.3)	101 (6.7)
200	87 (6.6)	96 (2.5)
500	92 (0.9)	97 (6.3)
2000	94 (3.0)	91 (3.1)

Table 2
Accuracy and precision of NE analysis in rat plasma (spiked NE concentration=200 pg/ml; 100 μl sample volume; $n=3$)

Analysis day	% Recovery (RSD, %)
1	110 (10.9)
2	101 (9.8)
3	88 (2.8)
4	108 (7.0)
5	94 (2.9)

used for day 1 and day 2 studies were found to have basal NE levels of 33±2.7 and 13.4±1.6 pg/ml, respectively. The lower limit of quantitation (LLOQ) was set at 10 pg/ml when using a 100-μl sample volume. However, due to the selectivity of the method the LLOQ could be easily lowered by using a larger sample volume or a smaller final reconstitution volume.

Various lots of blank rat plasma, however, were found to have basal NE levels ranging from 200 to 2500 pg/ml (data not shown). In order to use the blank rat plasma for spiking studies it was allowed to sit at room temperature until the endogenous NE dropped below the 100 pg/ml level. The results obtained for analyzing rat plasma (100 μl) spiked with 200 pg/ml NE ($n=3$) on five separate days are shown in Table 2. In general, the average accuracy was within ±10% of the target value, with RSD values being less than 10%. Additionally, due to the sensitivity and selectivity of the method, a study was run to determine the minimum sample volume that could be used for obtaining accurate NE measurements. Small volumes, 5, 10 and 25 μl, of a rat plasma sample spiked with 200 pg/ml NE were taken for analysis. The results of analyzing each sample are shown in Table 3. The NE values were within ±12% of target, although the RSD values obtained for the 5 μl sample was somewhat higher than observed with the larger sample volumes.

The methodology was found to be relatively robust over a 1 year period. Routine preventative maintenance of the GC and the MS instrumentation allowed NE analysis to be run on a continuous basis without difficulties over this time period. Preventative GC maintenance consisted of replacing the septum, injection port liner and removing approximately 30 cm from the front of the column after every 200 injections. Maintenance for the mass spectrometer consisted of cleaning the ion-volume after every 200 injections, cleaning the source as needed (typically after 1000 injections) and tuning on a monthly basis.

3.6. Animal study

The effects of a centrally acting α_2-agonist, clonidine, on heart rate, mean arterial pressure and plasma NE levels in SHRs are shown in Fig. 7. The administration of saline had no effect on any of the measured variables, while the administration of clonidine produced significant reductions in all of the measured parameters at all post-dosing time points with respect to the pre-dose values. Clonidine treatment also produced significant reductions in all measured variables with respect to the saline-treated control animals at the 30 and 60 min post-dose time points. The effects of clonidine relative to control animals had begun to wane at the 90 min post-dose time point; an effect expected due to the relatively short half-life of this agent in the rat. Importantly, there were no increases in heart rate, mean arterial pressure or plasma NE levels in the saline-treated group at any time point, suggesting that multiple, but small volume, blood sampling did not elicit a reflex increase in the sympathetic nervous system activity secondary to volume depletion. In fact, there was a tendency towards decreased heart rate, mean arterial

Table 3
Recovery of NE using variable rat plasma volumes ($n=3$)

Sample volume (μl)	Concentration NE (pg/ml)	% Recovery (RSD, %)
5	200	101 (14.8)
10	200	112 (6.9)
25	200	95 (4.7)

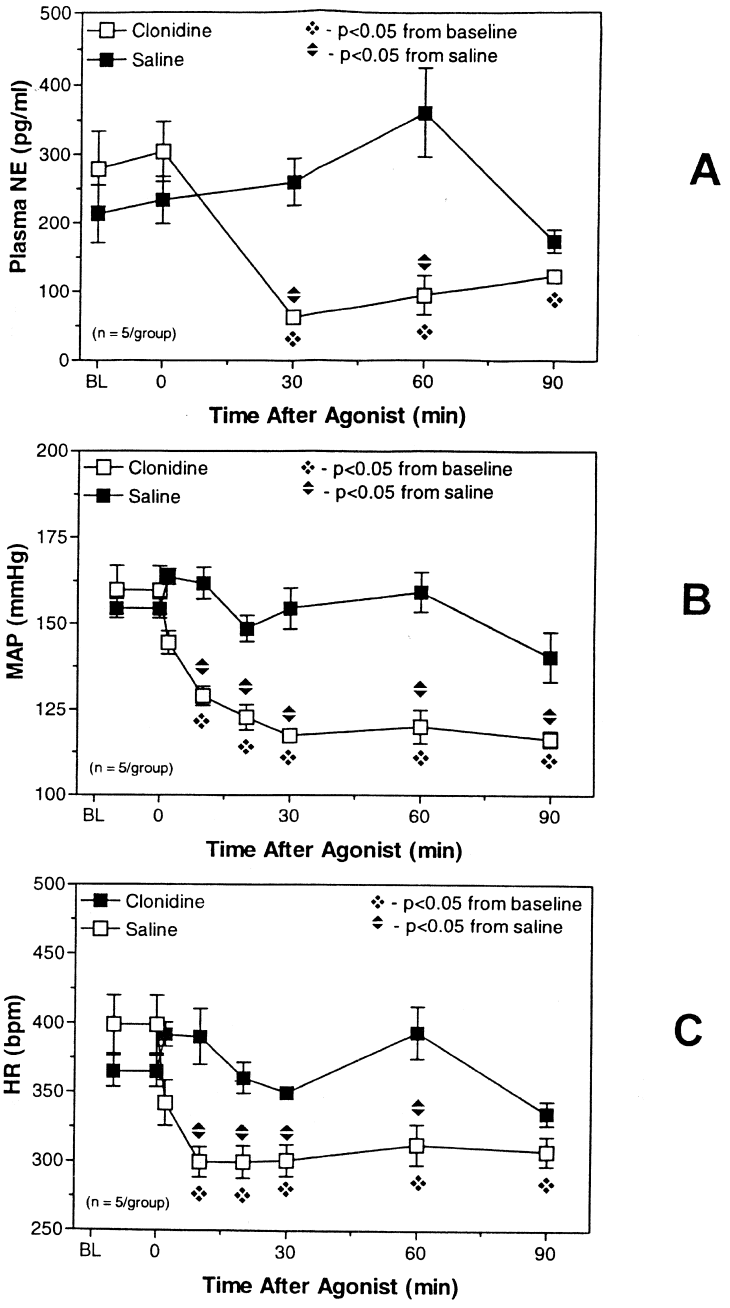

Fig. 7. Effect of clonidine on: (A) plasma norepinephrine (NE), (B) mean aterial pressure (MAP) and (C) heart rate (HR) in conscious spontaneously hypertensive rats. After animals were allowed about 20 min to acclimate to the attachment of catheters, a blood sample was taken (BL) and an injection of saline was given. At 15 min after the saline injection a second blood sample was taken (time 0). Immediately after the blood sample an i.v. injection of clonidine (open symbols) or saline (closed symbols) was administered. Note that the initial administration of saline had no effect on any of the measured parameters in either group. Note also that clonidine produced significant reductions in all the measured variables at all post-dose time points with respect to pre-drug values. Clonidine treatment also produced significant reductions in all measured variables with respect to saline-treated control animals at the 30 and 60 min post-dose time points.

pressure and plasma NE values in saline-treated animals during the 2 h experimental period. The decline is consistent with the well-known diurnal variation in plasma NE levels, with levels declining as the day progresses and rising again rapidly in the evening in this nocturnal animal [30].

4. Conclusions

A sensitive and selective stable-isotope-based GC–MS–MS–NICI method was developed for the analysis of NE in both rat and dog plasma. The methodology allowed the analysis of NE in small volumes of plasma, ranging from 5 to 100 μl, on a routine basis over a 1 year period. NE was demonstrated to be unstable in rat plasma at room temperature and the degradation of NE was shown to be blocked by the addition of semicarbazide to the whole blood during sample collection. The ability to measure trace NE levels in small plasma volumes allows for multiple samplings from the same animal during a short time period without disturbing the basal condition of the animal.

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