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Rapid and selective method for norepinephrine in rat urine using reversed-phase ion-pair high-performance liquid chromatography-tandem mass spectrometry

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Abstract

A rugged, high-throughput HPLC–MS–MS-based method, suitable for quantitation of norepinephrine (NE) in urine, has been developed. A rapid, batch-mode procedure utilizes alumina to isolate NE and its deuterated internal standard from urine. After release of NE, using dilute formic acid, samples are analyzed by isocratic reversed-phase ion-pair HPLC, with electrospray ionization (ESI) and MS–MS detection. The ion-pair reagent, heptafluorobutyric acid, is compatible with the ESI interface and permits use of mobile phases with relatively high methanol content, enhancing ESI sensitivity. Furthermore, no significant drop in sensitivity is observed throughout more than 15 h of instrument operation. The selectivity of this approach permitted simplification of the extraction procedure and reduced run times (under 4 min), making single batch-run sizes of more than 200 samples practical. The lower limit of quantitation is 5 ng per 0.5 ml sample, with analytical recoveries of 97–100% and overall method precision of better than 4% relative standard deviation verified up to 500 ng ml^{-1}. This method was initially applied to study the diurnal rhythm in sympathetic nervous system activity of spontaneously hypertensive rats.

Keywords: Diurnal rhythm; Norepinephrine; Noradrenaline; Heptafluorobutyric acid

1. Introduction

Norepinephrine (NE, Fig. 1) is a mediator of sympathetic nervous system (SNS) activity. As such, the ability to measure NE at low levels in biological matrices is often useful in the diagnosis and monitoring of certain disease states, for monitoring SNS side-effects of various drug therapies, in mechanistic studies, and as a tool for screening potential new drugs that may directly or indirectly affect the SNS. Previously reported methods for measuring NE in urine rely on multistep isolation procedures, followed by HPLC separation. Detection is most often by electrochemical techniques [1,2], although natural fluorescence [3,4], fluorescent derivatives [5], chemi-

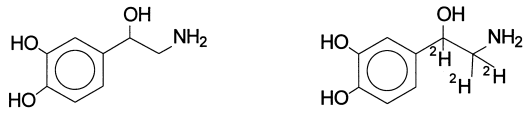

Fig. 1. Chemical structures of norepinephrine (NE) and $^{2}H_{3}$-norepinephrine (d-NE).

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luminescence [6], and phosphorescence [7] are also used. Radioimmunoassay [8,9] and radioenzymatic [10] procedures have also been tried. While these approaches are effective, each suffers from either sub-optimal selectivity, low throughput, and/or ruggedness concerns.

We have developed and describe here a more rapid and rugged, high throughput stable-isotope-dilution-based HPLC–MS–MS method, suitable for low ng ml^{-1} quantitation of NE in urine. This rapid, batch-mode procedure utilizes alumina to isolate NE and its $^{2}H_{3}$-labeled internal standard (d-NE, Fig. 1) from urine. After release, using dilute formic acid, NE and d-NE are then analyzed using isocratic reversed-phase ion-pair HPLC, with electrospray ionization (ESI) and selected-reaction-monitoring (SRM) MS–MS detection. In the SRM detection scheme, a parent ion is isolated by the first quadrupole and undergoes collisionally activated dissociation (CAD) in the second quadrupole. A resulting daughter ion, characteristic of the analyte of interest, is then isolated by the third quadrupole for detection. The selectivity of this approach permitted simplification of the extraction procedure and a reduction of run time to under 4 min, making single batch-run sizes of more than 200 samples practical. Special emphasis was also placed on assessing the compatibility of the heptafluorobutyric acid (HFBA) ion-pair reagent with the ESI-MS interface. In net, HFBA permits use of mobile phases with relatively high methanol content, enhancing ESI sensitivity, without a significant reduction in sensitivity throughout many hours of instrument operation.

2. Experimental

2.1. Chemicals and reagents

NE free base was obtained from Lancaster Synthesis (Windham, NH, USA) and d-NE free base was obtained from Isotec (Miamisburg, OH, USA). *n*-HFBA (HPLC grade) was purchased from Pierce (Rockford, IL, USA) and 80–200 mesh chromatography grade alumina was purchased from EM Science (Gibbstown, NJ, USA). Trizma buffer (tris-hydroxymethylaminomethane, pH 7.8) was obtained from Sigma–Aldrich (St. Louis, MO, USA). The formic acid (reagent grade) and methanol (HPLC grade) were purchased from J.T. Baker (Phillipsburgh, PA, USA) and used without further purification.

2.2. Urine sample collection

Spontaneously hypertensive rats (SHR) were obtained from Charles River Laboratories. Animals were housed in metabolism cages for collection of 24 h urine samples under a 12–12 h light–dark cycle (with lights coming on at 6 a.m. and going off at 6 p.m.). Because NE is readily oxidized (both chemically and enzymatically), urine samples were collected under stabilizing conditions. Oxidation was minimized by collecting the urine (from 0–7 ml) into cooled vials using refrigerated fraction collectors adapted to fit under the outlet of each metabolism cage. Each vial contained 200 μl of 6 *M* HCl when placed in the fraction collector so that urinary pH was maintained below 3. Urine samples were in four 6 h aliquots so that diurnal variation in norepinephrine excretion could be quantitated. All procedures involving the use of animals were carried out in an AALAC-accredited animal care facility and were pre-approved by the Institutional Animal Care and Use Committee of the Procter and Gamble Company. Urine samples were stored at −70°C prior to analysis.

2.3. Preparation of stock and working standard solutions

Stock solutions of NE and d-NE were each prepared at 1 mg ml^{-1} by addition of 10 mg free-base powder to a volumetric flask and diluting to 10.0 ml with 1 *M* HCl (aq). These solutions were stored at −20°C until needed to prepare working standard solutions. Each day of analysis, working standards of NE and d-NE were prepared by serial dilutions of stock solutions with 0.1 *M* formic acid (aq). Typically, d-NE was prepared at 10 μg ml^{-1}, while NE working standards were prepared at 5 and 0.5 μg ml^{-1}.

2.4. Preparation of calibration standards

Because NE is present at various levels in all urine samples, use of NE-spiked urine to generate cali-

bration curves was impractical. Non-matrix working standards were, therefore, prepared by combining 50 μl of d-NE working internal standard solution (500 ng d-NE) with various volumes of either of the two NE working standards (typically covering a 5–500 ng NE range), and diluting each combined solution to 1.00 ml with 1 *M* formic acid (aq).

2.5. Preparation of NE-spiked urine control samples

Urine control samples were prepared and analyzed to verify method accuracy and precision. To 500 μl aliquots of pooled, acid-preserved, rat urine was added 50 μl of d-NE working standard solution (500 ng d-NE), followed by known amounts (25, 100, and 250 ng) of NE. These control samples, along with blank (d-NE-only) rat urine were prepared in replicate ($n=6$), then extracted and analyzed, as described below. A composite endogenous NE concentration for the pooled rat urine was determined as the mean of measured values from the blank urine controls. This baseline NE level was then subtracted from values determined for NE-enriched control samples, providing a means to assess overall method accuracy and precision.

2.6. Extraction procedure

While our initial approach was to simply dilute and analyze the urine, incorporating no analyte isolation procedure, we found an unacceptable level of quenching of the ESI-MS–MS signal, presumably due to coeluting components in the urine. Therefore, currently available extraction technologies [1] were adapted to provide a modest degree of sample cleanup. A 500 μl aliquot of each urine sample was spiked with 500 ng of d-NE (50 μl of 10 $\mu g\,ml^{-1}$ d-NE working standard solution). Activated alumina was then added to each sample, using a custom-made glass scoop, roughly calibrated deliver approximately 50 mg of powder. One milliliter of 1 *M* Tris buffer (pH 7.8) was then added to each vial to raise the pH. Each sample tube was then capped and gently rocked for 10 min, allowing NE to adsorb to the alumina. Sample tubes were then centrifuged and the supernatants aspirated to waste. Five milliliters of water was then added to each tube, which was subsequently Vortex mixed to wash the alumina. The tubes were again centrifuged and the supernatant water aspirated to waste. One milliliter of 1 *M* formic acid (aq) was then added to each tube to desorb the NE. Sample tubes were rocked for 10 min and centrifuged once again. Approximately 200 μl of each supernatant was transferred into an autosampler vial for analysis.

2.7. Absolute recovery of NE from extraction procedure

An additional experiment was conducted to determine absolute recovery of NE from the alumina extraction procedure. One set ($n=6$) of blank (d-NE-only) urine control samples was prepared, as described above. A second set ($n=6$) of blank (d-NE-only) urine control samples was prepared identically, except that the d-NE was added after the extraction procedure. Both sets of prepared samples were then analyzed in a single HPLC–MS–MS batch-run. Thus, the mean NE/d-NE peak area ratio determined for the second set, divided by the mean ratio obtained for the first set of blank urine control samples, yields the fraction of NE recovered.

2.8. HPLC–MS–MS conditions

Liquid chromatography was performed using a Waters 600-MS HPLC system (Milford, MA, USA), in conjunction with a Gilson 234 autosampler (Middletown, WI, USA). A DuPont Zorbax Rx-C_8 (150× 2.1 mm I.D., 5 μm) column (MAC-MOD Analytical, Chadds Ford, PA, USA) was employed in an isocratic, reversed-phase ion-pair mode. The mobile phase consisted of water–methanol–HFBA (85:15:0.13, v/v/v), with a flow-rate of 0.3 $ml\,min^{-1}$. Sensitivity was such that only 5 μl of each sample was injected for analysis, representing 0.5% of the final solution. The entire chromatographic effluent was passed into the mass spectrometer interface for subsequent detection. Under these conditions, NE retention time was about 2.5 min, resulting in a total run time (injection-to-injection) of 3.5 min.

The mass spectrometer was a PE-Sciex API-III$^+$ triple quadrupole instrument (Concord, Ontario, Canada) operated in the TurboIonSpray configuration, consisting of the articulated IonSpray inlet used in conjunction with the heated TurboProbe desolvation unit. The TurboProbe temperature and nitrogen

gas flow-rate were 520°C and 8.0 l min^{-1}, respectively, and the nebulizer gas pressure was 300 kPa (nitrogen). Protonated analyte ions were generated using ESI and orifice potentials of 3500 and 50 V, respectively. The MS–MS detection scheme utilized CAD and SRM. CAD was accomplished using argon as the collision gas, at a thickness of 280×10^{12} molecules cm^{-2}, and an ion energy of 20 eV. The SRM transitions, m/z 170→107 and m/z 173→137, were sequentially monitored for detection of NE and d-NE, respectively. Dwell time for each transition was 200 ms.

2.9. Quantitation of NE

NE/d-NE area ratios were determined for the SRM chromatographic peaks using the PE-Sciex software package, MacQuan (version 1.4). Calibration curves were constructed by plotting peak area ratios (NE/d-NE) obtained for working standards versus NE concentration and fitting these data to a weighted $(1/x)$ linear regression curve, within the MacQuan software package. NE concentrations in test samples were then interpolated from this line.

3. Results and discussion

3.1. Absolute recovery of NE

The absolute recovery of NE from the alumina extraction process was determined by comparing NE/d-NE SRM chromatographic peak area ratios obtained for baseline rat urine samples, post- versus pre-extraction-spiked with d-NE. Recoveries were found to be 68.6±3.4% ($n=6$), from 500 μl baseline urines, estimated to contain 35 ng of endogenous NE in this case.

3.2. ESI mass spectra

The ESI mass spectra obtained for NE and d-NE are shown in Fig. 2. These spectra are characterized by an intense protonated molecular ion at m/z 170 and 173 for NE and d-NE, respectively. The two spectra are identical, except for the m/z shift due to the mass differences between NE and its stable-isotope-labeled analog. The daughter mass spectra obtained for NE and d-NE, following CAD of their respective protonated molecular ions, are shown in Fig. 3. The daughter spectra of both NE and d-NE contain several prominent fragment ions, with retention of two or more of the deuterium labels on several fragments. These data, combined with subsequent in-practice observations of chemical noise associated with each possible SRM scheme, led to selection of m/z 170→107 and m/z 173→137 as SRMs for detection of NE and d-NE, respectively. Note that it was necessary to monitor the less sensitive m/z 137 d-NE daughter ion (not analogous to the m/z 107 daughter ion of NE) to avoid otherwise high chemical background signal from the mobile phase in that SRM channel.

3.3. Chromatographic SRM profiles of standards and pooled rat urine

While the selectivity of MS–MS detection minimizes the need for clean chromatographic separations, some retention is required, if only to separate the analyte from numerous polar compounds and salts, eluting within (or immediately following) the solvent front. The highly polar nature of NE requires the use of an ion-pair reagent to get suitable retention on reversed-phase HPLC columns [11–13]. We found 10 m*M* HFBA to be a large, but ESI-MS-compatible, ion-pair reagent.

As examples, HPLC–MS–MS SRM chromatographic profiles for a 0 ng working standard, a 5 ng working standard, and a prepared blank rat urine control sample, are shown in Fig. 4a–c. Using the reversed-phase ion-pair approach, NE eluted from the column in about 2.5 min, with a k value of about 3.0 and with a reasonably symmetrical peak shape. Even under these rapid analysis conditions, NE was readily detectable in rat urine, with no interferences. This 5 ng standard represents 25 pg of NE on column (Fig. 4b). The urine control sample was shown to contain 57 ng of NE, reflecting an original urine NE concentration of 114 ng ml^{-1}. Factoring in the mean absolute NE recovery of 68.6 %, this NE SRM signal corresponded to an estimated 195 pg of NE on column (Fig. 4c).

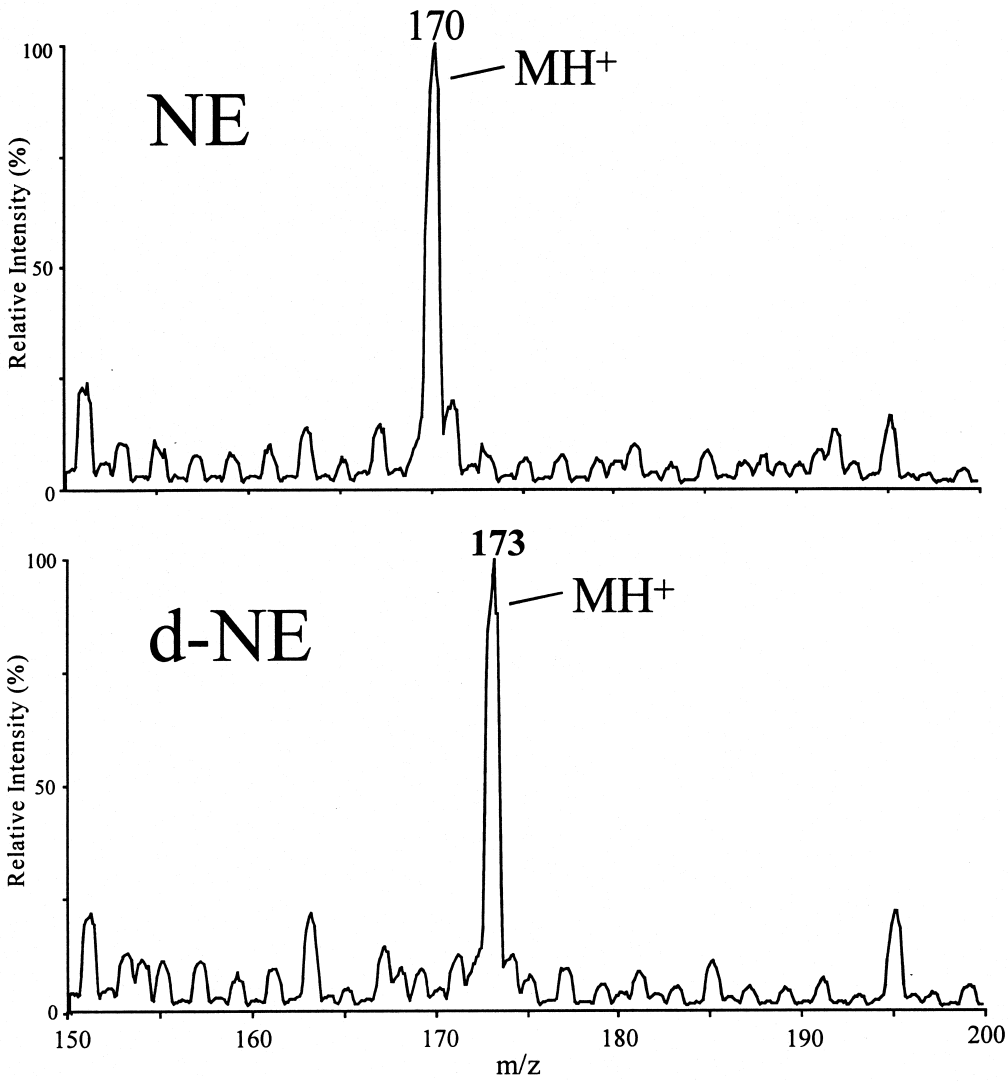

Fig. 2. Electrospray ionization mass spectra of: NE (top) and d-NE (bottom).

3.4. Standard curve

Standard curves were linear over the required calibration range (5–500 ng NE/sample), with correlation coefficients for the weighted ($1/x$) linear regression curves typically greater than 0.999. Replicate ($n=6$) injections of a 25 ng working standard resulted in R.S.D. values of less than 2% for NE/d-NE SRM peak area ratios. The S/N ratio obtained for the 5 ng working standard was typically greater than 20, which represented an adequate lower limit of quantitation for our rat urine applications.

3.5. Analysis of NE-spiked rat urine control samples

The accuracy and precision data from the HPLC–MS–MS analysis of rat urine control samples, obtained throughout a typical 200 sample batch run, are presented in Table 1. In this example, results from

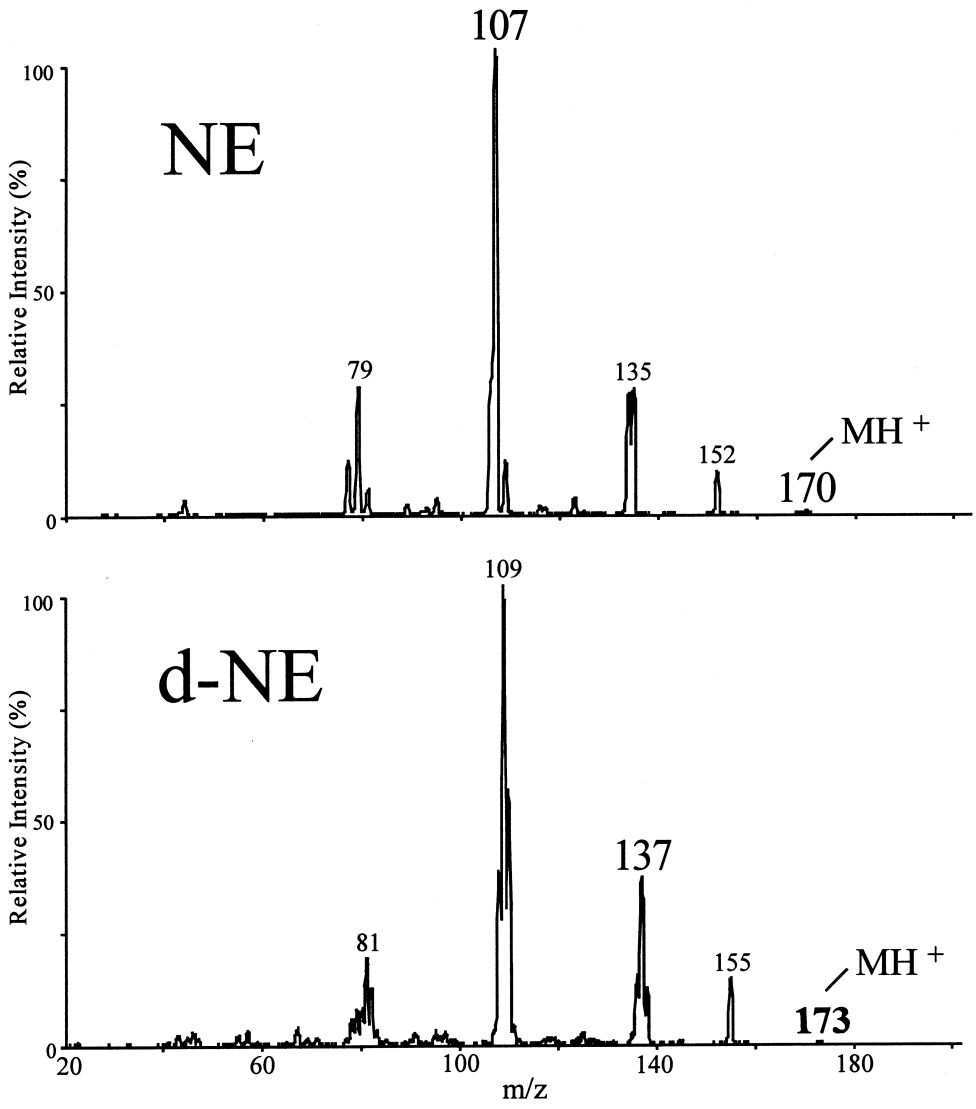

Fig. 3. Electrospray ionization tandem mass spectra (daughters-of-MH^+) of: NE (top) and d-NE (bottom).

analysis of the blank (d-NE-only) rat urine samples yielded a composite endogenous NE concentration for the pooled rat urine of 38.5 ng/500 μl. Subtraction of this value from the 'Total NE measured' value at each 'NE spiked' level led to the 'Spiked NE analytical recovery (%)' results.

Method accuracy across our range of interest was within 3% of the target value. Precision, as assessed by the R.S.D. for replicate analyses, was better than 4% at all levels.

3.6. HFBA as an ESI-MS-compatible modifier

A unique aspect of this method is the use of HFBA as the ion-pair reagent for ESI-MS-based detection. This modifier was key to our success in two ways: (1) It resulted in adequate HPLC retention and excellent peak shape for NE, even with moderate mobile phase methanol levels (NE is not retained at all, even at less than 5% methanol, without HFBA); and (2) Higher mobile phase methanol levels im-

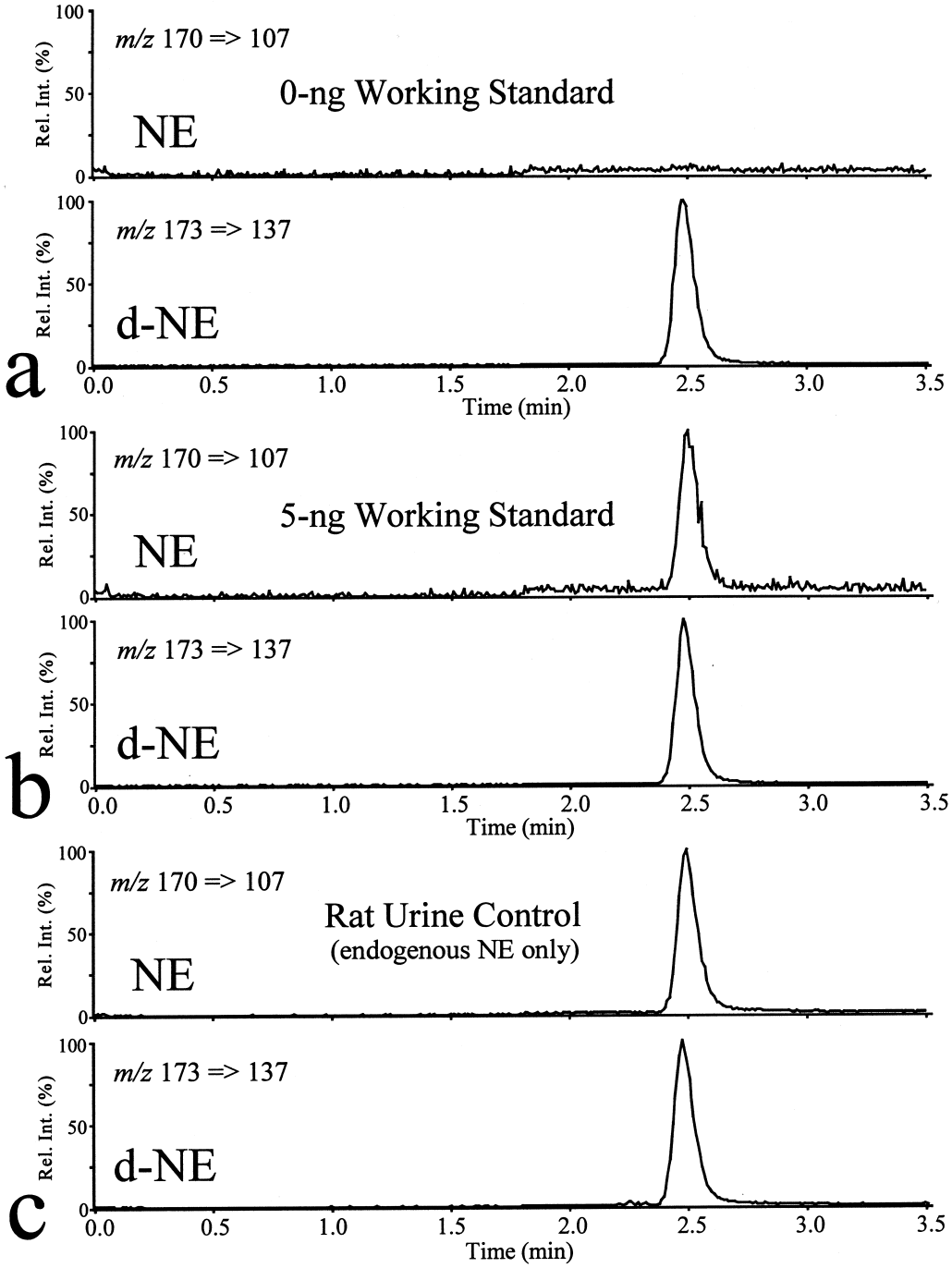

Fig. 4. HPLC–MS–MS SRM chromatographic profiles corresponding to NE (top) and d-NE (bottom) from analysis of: (a) 0-ng working standard (d-NE-only added); (b) 5-ng working standard (d-NE plus 5 ng/sample NE added); and (c) prepared 500 μl blank (d-NE-only added) rat urine control sample. The 5 ng working standard signal corresponds to 25 pg of NE on column.

Table 1
Accuracy and precision results from NE-spiked rat urine control sample analyses

NE spiked (ng)		*n*	Total NE measured (ng)	Spiked NE[a] measured (ng)	Spiked NE analytical recovery (%)	R.S.D. (%)
Blank	(0.0)	6	38.5	0.0	N/A	2.8
	25.0	6	63.4	24.9	99.6	3.0
	100.1	6	136.3	97.7	97.7	3.4
	250.3	6	282.4	243.8	97.4	2.5

[a] Calculated as total NE measured, less 38.5 ng, mean composite endogenous NE amount per 500 μl pooled urine sample.
N/A=Not applicable.

prove the efficiency of the ESI process relative to predominantly water-base mobile phase systems, enhancing method sensitivity. However, use of this sizable modifier (molecular weight 214) raised initial concerns pertaining to the impact it may have on instrument performance and method ruggedness. Fortunately, we have found that use of modest HFBA levels (10 m*M*) has no adverse effects, even over extended periods of method operation. This is demonstrated in Fig. 5, where internal standard (d-NE) peak areas for control urine samples are tracked over the course of a typical 13 h batch run (well over 200 injections). Other than reasonable sample-to-sample variability in absolute SRM signal (readily corrected for using the stable-isotope-labeled internal standard), there is clearly no discernible loss in instrument sensitivity throughout the course of a batch run. These results further endorse the suitability of 10 m*M* HFBA as an ESI-MS-compatible mobile phase modifier for trace-level quantitation.

3.7. Application to pilot drug study in rats

The utility of this method was demonstrated in a study of the diurnal rhythm in norepinephrine excretion in spontaneously hypertensive rats. In this study, six rats were housed in metabolism cages and given free access to drinking water. Four 6-h urine collections were made over a 24-h period. Each urine sample was then prepared and analyzed to determine NE concentration. This information, combined with associated urine volume data, provided a measure of total urinary NE excretion per each 6 h sampling period for each study animal. Net results of this

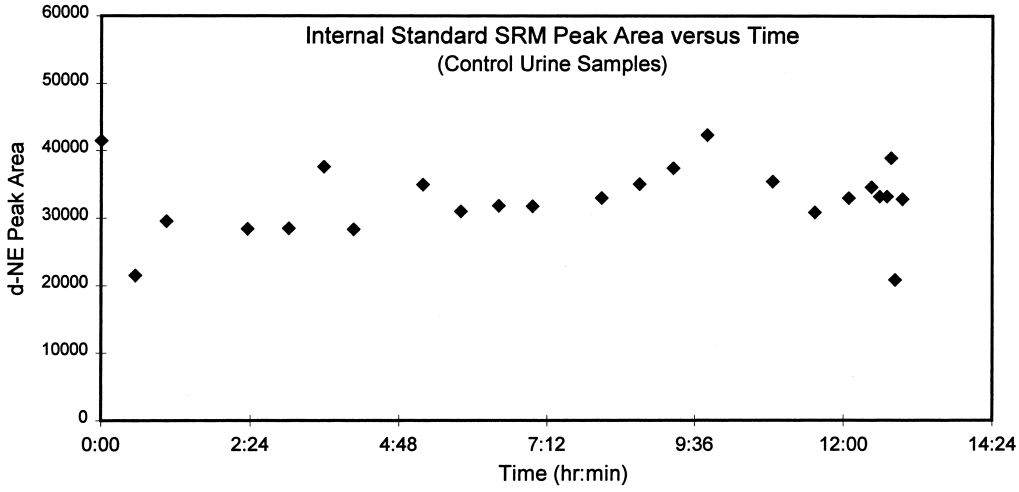

Fig. 5. Plot of internal standard (d-NE) SRM peak areas versus time, for control urine samples dispersed throughout a 13-h HPLC–MS–MS batch run (over 200 injections).

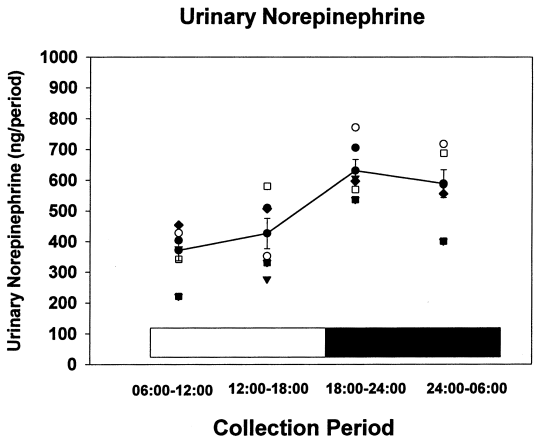

Fig. 6. Urinary NE excretion per 6 h from rats obtained at different times during a 12 h light–dark cycle. The timing of each collection period was as shown on the graph. Lights in the animal rooms came on at 6 a.m. and went out at 6 p.m. (18:00). Note that there was a highly significant increase in urinary NE excretion during the 'lights out' periods ($P<0.002$, one-way ANOVA).

study are shown graphically in Fig. 6. Note that there was a statistically significant increase ($P<0.002$; one-way ANOVA) in the urinary NE excretion during the two collections obtained during the dark part of the light/dark cycle. This corresponds to the period of highest motor and sympathetic nervous system activity in the rat, a nocturnal animal, as reported by others [14].

4. Conclusions

A rapid and accurate HPLC–MS–MS method for measuring NE in rat urine has been developed. This method required a less rigorous sample preparation than described in previous literature and, due to the deuterated internal standard, was less susceptible to minor variations in the extraction process. The procedure also required no sample derivatization. The selectivity of MS–MS detection allowed run times of under 4 min per sample, with sharp HPLC peaks and good sensitivity. Paramount to the utility of this approach, it was also demonstrated that HFBA is an effective ESI-MS-compatible ion-pair reagent, useful for increasing retention of highly polar compounds for quantitation by HPLC–MS–MS. This reagent was suitable for long batch runs, up to at least 13 h, allowing analysis of over 200 study samples.

Finally, it was demonstrated that urinary NE excretion rates were useful as an indicator of sympathetic nervous system activity in rats. This indirect relative measure of sympathetic tone offers a viable noninvasive alternative to more-direct blood NE measurements, which require blood drawing procedures that can momentarily and artificially elevate NE levels, potentially leading to erroneous assessments regarding the effect of treatment.

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