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TITLE

QUANTITATION OF EPINEPHRINE, NOREPINEPHRINE, DOPAMINE, METANEPHRINE AND NORMETANEPHRINE IN HUMAN PLASMA USING NEGATIVE ION CHEMICAL IONIZATION GC-MS

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Quantitation of Epinephrine, Norepinephrine, Dopamine, Metanephrine and Normetanephrine in human plasma using negative ion chemical ionization GC-MS.

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Résumé

La quantification de l'épinéphrine (EPI), la norépinéphrine (NEPI), la dopamine (DOP) et de la métanéphrine (NMET) dans du plasma sanguin humain par GC-MS avec EC-NCl en utilisant une mixture ammoniaque/méthane à 5 % en guise de gaz réactif, est décrite. Les standards internes utilisés sont le D_3-épinéphrine, le D_3-norépinéphrine ainsi que la D_2-dopamine. La D_3-épinéphrine a été utilisée pour le dosage de la MET et de la NMET. Les échantillons de plasma furent extraits à l'aide de tubes à échanges ioniques en phase solide, congelés puis dérivatisés à l'acide pentafluoropropionique anhydre. Les ions de quantification utilisés pour le monitoring sélectif étaient : EPI - m/z 767, D3-EPI - m/z 770, NEPI - m/z 753, D_3-NEPI - m/z 756, DOP - m/z 571, D_2-DOP - m/z 573, MET - m/z 635 et NMET - m/z 621. La sensibilité des dosages était suffisamment élevée pour permettre la mesure de ces amines biogènes dans 0.1 ml de plasma sanguin humain (les moyennes mesurées sont : EPI - 25 pg/ml ; NEPI - 280 pg/ml ; DOP - 33 pg/ml ; MET - 28 pg/ml ; NMET - 53 pg/ml). La méthode fut mise à l'épreuve sur 1000 échantillons et s'est avérée spécifique et reproductible.

Abstract

Quantitation of epinephrine (EPI), norepinephrine (NEPI), dopamine (DOP), metanephrine (MET) and normetanephrine (NMET) in human blood plasma by gas chromatography-mass spectrometry with electron capture negative ion chemical ionization (EC-NCI) using a 5% ammonia/methane mixture as a reagent gas is described. Deuterium labeled D_3-epinephrine, D_3-norepinephrine and D_2-dopamine were used as internal standards. D_3-epinephrine was used for quantitation of the MET and NMET. Plasma samples were extracted using ion exchange solid phase tubes, freeze dried and derivatized with pentafluoropropionic acid anhydride. The quantitation ions used for the selective ion monitoring were: EPI - m/z 767, D_3-EPI - m/z 770, NEPI - m/z 753, D_3-NEPI - m/z 756, DOP - m/z 571, D_2-DOP - m/z 573, MET - m/z 635 and NMET - m/z 621. The assay sensitivity was sufficient to measure resting concentrations of these biogenic amines in 0.1 mL of human blood plasma (the mean measured concentrations were: EPI - 25 pg/mL; NEPI - 280 pg/mL; DOP - 33 pg/mL; MET - 28 pg/mL; NMET - 53 pg/mL). The method was used to analyze over 1000 specimens and proved to be rugged, specific and reproducible.

Introduction

For our studies of the immune system of humans exposed to heat and exercise, we needed a sensitive and specific method for measurements of free epinephrine (EPI), norepinephrine (NEPI), dopamine (DOP), metanephrine (MET) and normetanephrine (NMET) in human blood plasma. Our prime objective was to use one assay which would: require a small volume of sample, be highly specific and efficiently prepare samples. The majority of available techniques were based on HPLC with an electrochemical detection (1-3). There were numerous reports dealing with applications of GC-MS using electron impact or positive ion chemical ionization for the analysis of various catecholamines and their acid metabolites in urine, blood plasma and vitreous humour (1,4-8). All above methods required 1-6 mL of sample, more than we were prepared to use, since we planned to analyze other compounds of interest in the same specimens. The large sample size and non-specificity of these methods stirred us to develop a simple and sensitive method for all of the above analytes, using solid phase ion exchange extraction and gas chromatography-mass spectrometry with electron capture negative ion chemical ionization (EC-NCI). Our method is based on work of Martin et al (9). These researchers looked at the feasibility of using pentafluoropropionic anhydride (PFPA) derivatization for GC-MS EC-NCI analysis of various O-methylated catecholamines. They developed a quantitation method for analyses of normetanephrine in cerebrospinal fluid. It is a well established practice to use perfluoroacylating reagents to enhance sensitivity for hydroxy- and amino- group containing compounds for analyses by gas chromatography with electron capture detection or by EC-NCI mass spectrometry. The sensitivity of the GC-MS EC-NCI technique facilitates use of smaller sample volume, and therefore allows for more frequent sampling of tested subjects. Using a smaller sample size also extends the life span of the expensive gas chromatographic columns, saves extraction solvents, etc. Our simplified method (10) required only 0.1 mL of human blood plasma sample to measure the resting concentrations of the five catecholamines (EPI, NEPI, DOP, MET and NMET) in one GC-MS injection. The assay has been used in our laboratory to analyze over 1000 human blood plasma samples.

Materials and Methods

Epinephrine (base), norepinephrine HCl, metanephrine HCl, normetanephrine HCl, dopamine HCl and ascorbic acid were purchased from Sigma Chemical Co., St. Louis, MO, USA. Pentafluoropropionic anhydride (PFPA), solid phase ion exchange WCX Supelclean extraction columns (1mL size), and a solid phase tube extraction vacuum manifold apparatus were obtained from Supelco Canada, Mississauga, ON, Canada. DL-Epinephrine (α,α,β-D_3), DL-Norepinephrine (α,α,β-D_3) and DL-Dopamine (2,2-D_2) were obtained from Cambridge Isotope Laboratories, Andover, MA, USA. BioRad Lyphochek Endocrine Control plasmas Level 1 and 2 were purchased from Bio-Rad Laboratories, Mississauga, ON, Canada. All solvents used were of analytical grade and were purchased from Fisher Scientific, Markham, ON, Canada. The 5% ammonia-methane gas mix was purchased from Matheson Co, Toronto, ON, Canada. The gas chromatographic Hewlett-Packard fused silica HP-5 columns (30 m × 0.25 mm, 0.25 μm) were obtained from Hewlett-Packard, Mississauga, ON, Canada.

The work was carried out on a Finnigan TSQ-700 GC-MS/MS equipped with a CTS-200s autosampler and a DECstation 5000 based ICIS II data system. The GC-MS conditions were as follows: injector 180°C, split valve opened 45 sec. after injection; helium at 10 psi head pressure was used as a carrier gas; column temperature was held at 100°C for 1 min. then increased to 250°C at 15°C/min. and subsequently kept for 10 min. at 250°C; the GC/MS interface temperature was kept at 260°C. The mass spectrometer was used in the negative ion chemical ionization mode, using a methane/ammonia mixture (95:5)

as the chemical ionization reagent gas (11). The mass spectrometer was tuned for the NCI operation, calibrated, and the ion source pressure was optimized, using the PFTBA base peak fragment m/z 633. The ion source was kept at 120°C. The filament emission current was 200 mA, the electron multiplier was set at 1400 V and the electrometer gain was set at 10^8. Selective ion monitoring was done using the first quadrupole mass analyzer. Monitored fragments were as follows: m/z 770 - D_3-epinephrine; m/z 767 - epinephrine; m/z 756 - D_3-norepinephrine; m/z 753 - norepinephrine; m/z 573 - D_2-dopamine; m/z 571 - dopamine; m/z 621 - normetanephrine; m/z 635 - metanephrine. The dwell time was set to 10 msec/ion channel.

Catecholamines (base or various salts) are insoluble in most organic solvents, sparingly soluble in methanol or ethanol but are soluble in acidified water (pH<5); therefore, all stock solutions of non labeled and deuterium labeled materials were prepared in 0.1% ascorbic acid aqueous solutions. The stock solutions were kept at 6°C sealed under N_2. Human blood plasma samples (prepared with EDTA) were kept at –70°C and were analyzed within few weeks after collections. The deuterium labeled D_3-epinephrine, D_3-norepinephrine and D_2-dopamine were used as internal standards for the quantitations. For metanephrine and normetanephrine, D_3-epinephrine was used as an internal standard for all calculations. Four-point calibration standard curves were prepared by spiking saline solutions with 10, 100, 500 and 1000 pg/mL of epinephrine, metanephrine, normetanephrine and dopamine and 100, 500,1000 and 5000 pg/mL of norepinephrine. Catecholamine standards and plasma samples (100 μL) were pipetted into silanized conical test tubes followed by the internal standards (10 pg of D_3-epinephrine and D_2-dopamine, together with 100 pg D_3-norepinephrine combined in 100 μL solution of 0.1% of ascorbic acid). Aliquots of human plasma pool and BioRad Lyphochek low and high endocrine control plasmas (100 μL) were also analyzed as quality control samples with every batch of 24 samples. The calibrators, controls and samples were extracted and purified on preconditioned ion exchange Supelco WCX Supelclean 1mL columns using a Supelco vacuum manifold apparatus. The column preconditioning was done according to the manufacturer's recommendations with 0.5 mL of 0.5N HCl and 2 mL of distilled water. All test tubes were gently vortexed. Plasma samples were treated with 0.1 mL of saturated cadmium chloride solution, spun at 2500 rpm for 5 minutes to separate the precipitated proteins. The supernatants were transferred onto preconditioned solid phase columns and washed with 3 mL of distilled water. Care was taken in all the steps not to let the solid phase extraction columns go dry. Catecholamines were slowly eluted (over 3 min.) with 0.3 mL of 0.1 M formic acid and 0.1 mL of distilled water, snap frozen and freeze dried overnight. The vacuum was gently broken with N_2 gas from an inflatable balloon. The dried extracts were subsequently derivatized with 50 μL of pentafluoropropionic anhydride (PFPA), for 30 min. at 60°C. The excess of derivatization reagent was gently removed under a stream of dry nitrogen at room temperature; the dry residues were re-dissolved in 25 μL of toluene. The solutions were transferred into 100 μL autosampler vials, and one microliter was injected into the GC/MS. The calibration curves were obtained using linear regression through 4 calibration points. Calculations and graphs were done with the Microsoft Excel spread sheet.

Results and Discussion

The cleanliness of the ion source, low source temperature, low ionization energy, plus choice and pressure of the reactant gas are major factors in setting up a sensitive EC-NCI mass spectrometric assay. There was a substantial increase in our catecholamine method sensitivity when we substituted pure methane CI reagent gas with a 5% ammonia/methane mix. The sensitivity of the EC-NCI mass spectrometric technique makes cross-contamination of samples a much more acute problem in comparison with the less sensitive PCI or EI techniques. The use of disposable silanized glassware and chromic acid wash of re-usable glassware is an absolute necessity. The major fragmentation pattern was the same for epinephrine-PFP, norepinephrine-PFP, metanephrine-PFP, and normetanephrine-PFP (Fig. 1A,1B,1D,1E). The major ion (base peak) was the negatively charged molecular ion $[M]^-$. Other prominent ions were $[M\text{-}HF]^-$ and $[M\text{-}C_2F_5CO]^-$. In the case of dopamine (Fig. 1C), the molecular ion was not observed and $[M\text{-}HF]^-$ was the base peak. Other strong fragments were $[M\text{-}2HF]^-$ and $[M\text{-}H\text{-}C_2F_5CO]^-$.

Since catecholamines are easily susceptible to oxidation, anti-oxidants such as ascorbic acid or sodium metabisulfide were used for the preparation of all internal standards, calibrators, quality controls and samples. The use of aqueous ascorbic acid eliminated the variability of results that was observed with the initial assay set up. The blood plasma samples collected and analyzed without addition of any anti-oxidants gave lower than expected normal resting plasma catecholamine concentrations. The highest degree of degradation was observed for epinephrine. It should be stressed again that a careful handling of samples to prevent an oxidative loss is an important factor in quantitative analysis of catecholamines. The blood plasma samples are much preferred over the blood serum samples. The plasma preparation

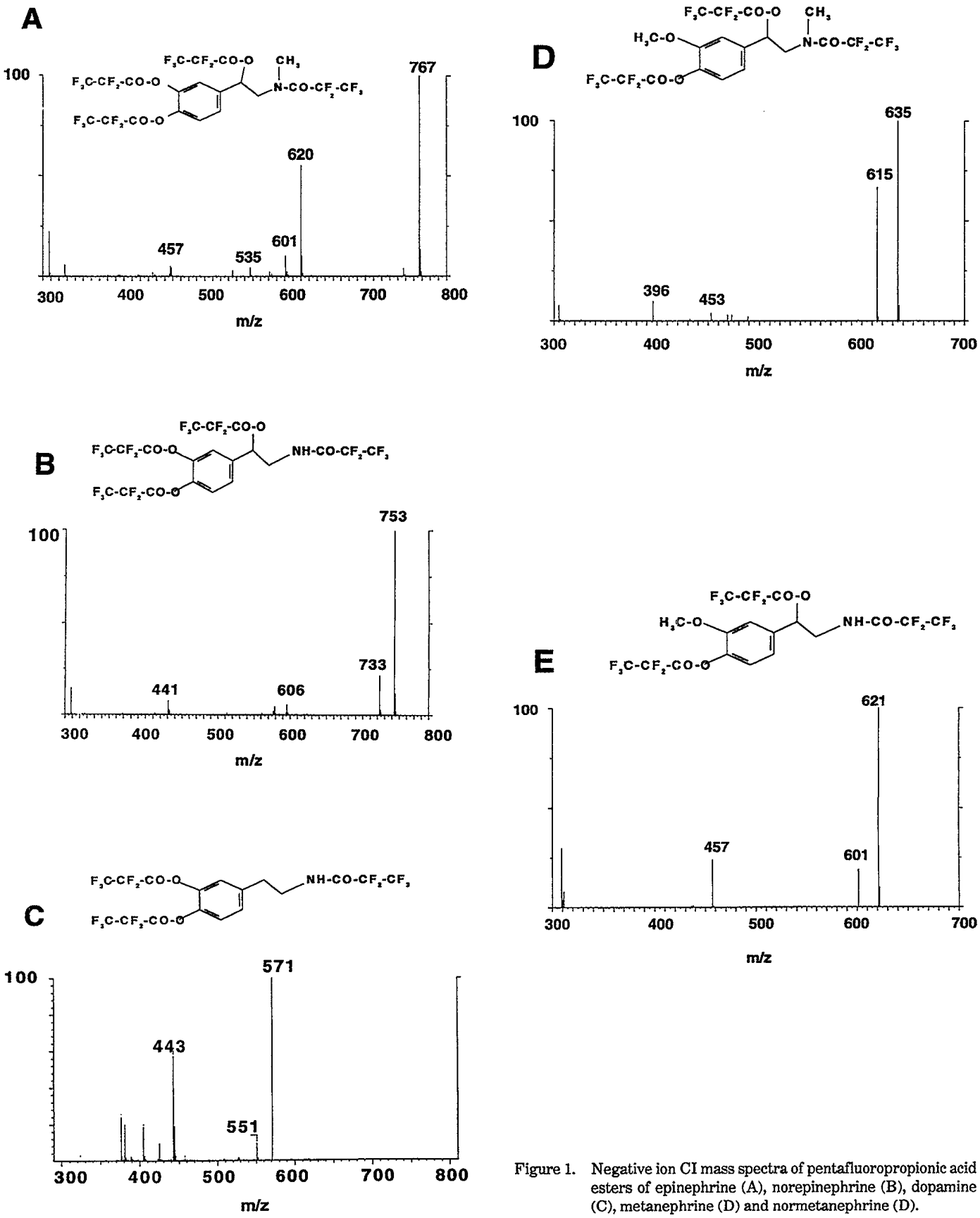

Figure 1. Negative ion CI mass spectra of pentafluoropropionic acid esters of epinephrine (A), norepinephrine (B), dopamine (C), metanephrine (D) and normetanephrine (D).

process, opposed to preparation of serum, can be fast and well controlled to prevent the loss of catecholamines due to the oxidation. A reasonable care had to be taken not to produce hemolysis of red blood cells. It was observed that recoveries of catecholamines and added internal standards diminished greatly with any increase of red coloration in tested samples. We believe that this loss was not attributable to an increased sample oxidation since any extra additions of various anti-oxidants did not improve the internal standard recoveries.

The assays' precision calculations were done for the Lyphochek endocrine control plasma samples and our pooled human plasma samples. Those data are presented in Table I. The resulting four point linear regression calibration curves had correlation coefficients (r) greater than 0.998. The inter assay reproducibility of calibration curves was also expressed as coefficients of variation of the calibration curve slopes (% CV; n=6; EPI - 2.25%; NEPI - 5.71%; DOP - 7.16%; MET - 9.85%; NMET - 10.21%). The solid phase extraction recoveries of catecholamines from plasma samples ranged from 75% to 85%. These calculations were based on a comparison of extracted and non-extracted internal standard areas. Since we did not have any deuterium labeled internal standards for metanephrine and normetanephrine at our disposal, their recoveries were assumed to follow the epinephrine recoveries, and D_3-epinephrine was used for their calibration curves and quantitative calculations. There were no major interfering signals in either of the monitored mass channels at the measured catecholamines retention time windows. A mass chromatogram of our control plasma pool sample is shown in Fig. 2. One mL aliquots of our plasma pool samples were spiked with 50 pg of EPI, DOP, MET and NMET and 100 pg of NEPI. The measured concentrations fell within 10% of the expected added endogenous and exogenous values.

The relatively narrow dynamic range inherent to all NCI methods can be easily overcome by volume adjustments of plasma aliquots. Since our method measured five catecholamines in a single plasma sample, the overall limit of quantitation for the method was dictated by the least concentrated component, epinephrine. The plasma volume size of 0.1 mL was selected as a compromise that allowed us to reliably measure the low resting

Table I. Precision study for intra- assay and inter- assay: mean concentration [pg/mL], standard deviation and coefficient of variation, using (a.) the BioRad Lyphochek Endocrine Control plasmas Level 1 and 2 and (b.) the laboratory control plasma pool.

(a) Intra Assay Precision (n=16)

	Epinephrine	Norepinephrine	Dopamine	Metanephrine	Normetanephrine
Low Control					
Mean [pg/mL]	123.9	322.03	85.0	-	-
SD	9.11	16.29	6.91	-	-
CV%	7.36	5.06	8.12	-	-
High Control					
Mean [pg/mL]	1340.5	1535.4	446.8	-	-
SD	57.54	41.86	21.24	-	-
CV%	4.29	2.73	4.75	-	-
Plasma pool					
Mean [pg/mL]	38.8	265.8	85.9	46.5	51.7
SD	3.62	24.99	7.76	5.45	5.52
CV%	9.32	9.40	9.04	11.72	10.68

(b) Inter Assay Precision (n=10)

	Epinephrine	Norepinephrine	Dopamine	Metanephrine	Normetanephrine
Plasma pool					
Mean [pg/mL]	40.0	264.4	84.3	44.5	53.8
SD	1.47	3.73	2.19	3.46	4.85
CV%	3.68	1.41	2.59	7.78	9.01

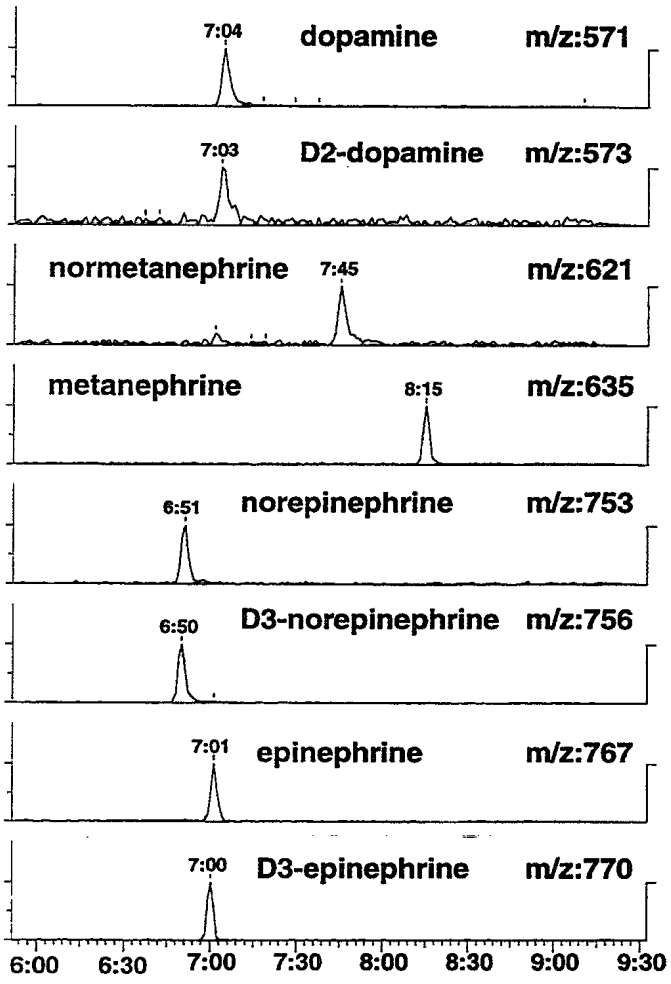

Figure 2. Mass chromatograms of DOP, D_2-DOP, NMET, MET, NEPI, D_3-NEPI, EPI and D_3-EPI extracted from the pooled control human blood plasma (Table 1b)

epinephrine concentrations (down to 10 pg/mL) as well as the highly elevated levels of norepinephrine found in other samples. Several samples with high concentrations of catecholamines caused a saturation of the ion source that resulted in flat top mass chromatographic peaks. These samples had to be diluted with toluene and re-injected.

The plasma specimens were processed as 2 batches of 24 tubes per day. Samples were kept frozen at –70°C and were derivatized on the day of GC-MS analyses. All quality control samples' concentrations had to fall within ± 10% of expected values or the sample batch was re-assayed. The method allowed us to process a large number of specimens in a relatively short time, with only routine GLC maintenance. In one of our studies, nine healthy male subjects were subjected to four testing sessions. Each individual served as his own control. The first session was set to establish the basal plasma catecholamine levels, at a room temperature with no exercise. The other three regimes looked at the plasma catecholamine concentrations of subjects exposed to heat (40°C), exercise at room temperature, and exercise in a hot environment. The mean concentrations of free catecholamines in the control resting group were as follows: 25 pg/mL - EPI; 280 pg/mL - NEPI; 33 pg/mL - DOP; 28 pg/mL - MET; 53 pg/mL - NMET. The highest average maximum levels observed in the case of heat and exercise stress were 230 pg/mL - EPI, 2200 pg/mL - NEPI, 120 pg/mL - DOP, 160 pg/mL - MET and 250 pg/mL - NMET (Fig. 3). Details with other immunological data and conclusions of the whole study will be presented elsewhere (12).

We have also tried to adapt the method to the recently acquired Finnigan GCQ Tandem ion trap. This mass spectrometer is capable of negative ion CI operation in a mass range 2 to 1000 amu. We have not been successful in achieving the required sensitivity to analyze plasma catecholamines with this instrument. To obtain mass spectra that were similar to the Finnigan TSQ-700 instrument spectra, we had to modify the setup conditions from the factory preset values and use low emission filaments. Even with these alterations, the sensitivity of our GCQ mass spectrometer was 100-1000 fold less for the plasma sample extracts than the sensitivity achieved with the quadrupole instrument. Our old TSQ-700, operating in the SIM EC-NCI mode, and injected on column with 25 femtograms of pure PFPA derivatized catecholamine standards, produced signal to noise ratios better than 10:1 for the five tested catecholamines. In the GCQ instrument, all the scanning functions are controlled through software. With the current 2.0 software version, the ion trap was not capable of achieving the expected mid-femtogram range. This may change in the future with newer software releases and hardware updates. The

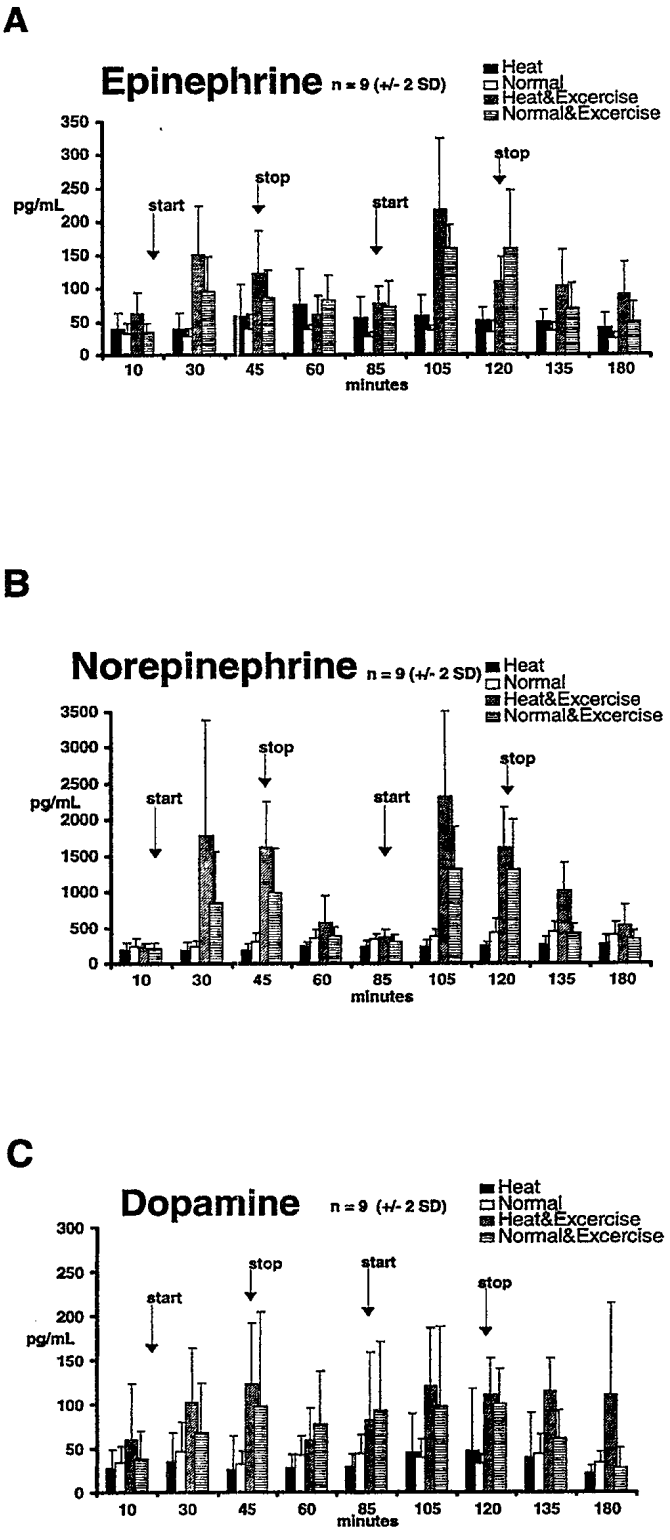

Figure 3. Plasma concentrations of epinephrine (A), norepinephrine (B) and dopamine (C) measured to assess levels of stress during repeated exercise at normal and high ambient temperatures.

ion trap scanning functions and conditions should be tailored to an individual compound requirement, since it seems that the sensitivity of ion traps is largely compound dependent.

In conclusion, the above EC-NCI GC-MS method for determination of blood plasma catecholamine concentrations is useful where high specificity and sensitivity are required and a limited volume of plasma samples is a major concern.

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