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SELECTED-ION MONITORING GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF CATECHOLAMINES: ENHANCEMENT OF SENSITIVITY BY A SIMPLE CLEAN-UP STEP ON SEPHADEX G-10

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SUMMARY

The sensitivity of catecholamines using selected-ion monitoring gas chromatography–mass spectrometry (SIM GC–MS) has been enhanced by employing a simple clean-up step using Sephadex G-10 columns. This procedure allows for the detection of extremely small amounts (1–10 pg) of biogenic amines (e.g., epinine) which corresponds to a 25-fold increase in detection limits compared to electron ionization GC–MS or high-performance liquid chromatography with electrochemical detection (HPLC–ED). The SIM GC–MS assay was used to monitor the dopamine-β-hydroxylase-mediated conversion of epinine d_6 to epinephrine d_6 in rat hypothalamus and brainstem in vitro and the results were compared to those obtained by HPLC–ED

INTRODUCTION

Selected-ion monitoring gas chromatography–mass spectrometry (SIM GC–MS) and high-performance liquid chromatography with electrochemical detection (HPLC–ED) have been used for the quantification of catecholamines in tissue and body fluids. Both methods were reviewed recently [1,2]. HPLC–ED is advantageous for routine measurements because it requires less laborious sample preparation and less expensive equipment. SIM GC–MS, on the other hand, provides highly specific information due to the monitoring of structurally diagnostic fragment ions. Electron capture negative-ion chemical ionization (NICI) GC–

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MS has been shown to be up to 200-fold more sensitive than other ionization techniques allowing the quantification of these compounds at the femtomole level [3,4].

Solid phase alumina extraction has been widely used to isolate catecholamines from body fluids and tissue [5–7]. In our laboratories, catecholamines extracted from rat brain by this method could be readily analyzed by HPLC–ED at the picomole level. However, when routine GC–MS measurements were carried out on these samples following published procedures [6,7], the analysis was plagued by peak broadening and high chemical background. Simultaneously, contaminant accumulation in the injector, especially on the injector tip, and relatively rapid column deterioration were observed.

In this paper we wish to report the improvement of a SIM GC–MS assay by a simple clean-up step using small Sephadex G-10 columns following alumina adsorption of catecholamines from brain tissue. This procedure enables the detection of extremely small amounts of biogenic amines (1–10 pg). The applicability and advantages of this assay for biosynthetic studies using deuterated precursors was investigated by comparing it with HPLC–ED.

EXPERIMENTAL

Materials and reagents

Chemicals were obtained from the following sources: norepinephrine, epinephrine, dihydroxyphenylacetic acid (DOPAC), dopamine hydrochloride, epinine hydrochloride, dihydroxybenzylamine hydrobromide (DHBA), pargyline and Sephadex G-10 from Sigma (St. Louis, MO, U.S.A.); pentafluoropropionic acid anhydride (PFPA) from Pierce (Rockford, IL, U.S.A.) and epinephrine-α,α,β-d_3 from Merck Sharp & Dohme Isotopes (Montreal, Canada). The 2 *M* hydrochloric acid in methanol was prepared from dry hydrogen chloride gas and anhydrous methanol. All other chemicals were obtained from commercial sources in the highest purity available. Solutions and buffers were made with water purified by the Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Synthesis of deuterated epinines

N-Trideuteromethyl-2-(3′,4′-dihydroxy-2′,5′,6′-trideuterophenyl)ethylamine (epinine-d_6) and N-trideuteromethyl-2-(3′,4′-dihydroxyphenyl)ethylamine (epinine-d_3) were synthesized as described earlier [8].

In vitro incubations

Rat brain areas were dissected and incubated as described in detail elsewhere [9]. Briefly, minced tissue was preincubated in physiological buffer (126.4 m*M* NaCl, 27.6 m*M* $NaHCO_3$, 2.4 m*M* KCl, 2.7 m*M* $CaCl_2$, 0.8 m*M* $MgCl_2$, 0.5 m*M* Na_2SO_4, 0.5 m*M* KH_2PO_4, 5.9 m*M* D-glucose; saturated with O_2–CO_2, 95:5) containing 100 μM pargyline for 15 min at 37°C. Epinine-d_6 in 0.005 *M* perchloric acid was added to the desired concentration to obtain a total final volume of 1 ml, and the samples were incubated for 20 min at 37°C. The reaction was stopped by addition of 100 μl of ice-cold 0.4 *M* perchloric acid containing 25 pmol DHBA or

25 pmol epinephrine-α,α,β-d_3 as the internal standards for HPLC–ED and SIM GC–MS analysis, respectively. Samples for the detection of endogenous epinine were incubated with pargyline only. Epinine-d_3 (2 pmol) served as the internal standard in these experiments. The samples were briefly sonicated and centrifuged at 15 600 *g* for 5 min. The supernatant was removed and the pellet was washed with 200 µl of physiological buffer and saved for biuret protein determination using bovine serum albumin as standard [10].

Extraction and clean-up procedure

About 30 mg of acid-washed alumina, prepared according to Anton and Sayre [5], and 400 µl of 0.5 *M* Tris buffer, pH 8.6, containing 5 g/l EDTA and 2 g/l sodium metabisulfite were added to the combined supernatant and was obtained as described in the previous paragraph. The tubes were mixed by inversion for 20 min at room temperature; the supernatant was discarded and the alumina washed three times with 1 ml water. The catecholamines were recovered in 100 µl of 0.1 *M* hydrochloric acid and then applied to small Sephadex G-10 columns (50 mm height) in Pasteur pipets that had been allowed to hydrate overnight in 0.02 *M* ammonium hydroxide and had been washed with 2 ml of 0.01 *M* hydrochloric acid prior to the experiment. The columns were washed with two 450-µl volumes of 0.01 *M* hydrochloric acid, and the washes were discarded. The basic catecholamines were eluted with two 500-µl volumes of 0.01 *M* hydrochloric acid and lyophilized. DOPAC was eluted within the following 1-ml fraction of 0.01 *M* hydrochloric acid. The columns could be reused after washing with two 2-ml volumes of 0.02 *M* ammonium hydroxide and 2 ml of 0.01 *M* hydrochloric acid. The columns could be stored in 0.02 *M* ammonium hydroxide.

Derivatization of catecholamines

The lyophilized extracts were heated in 1-ml silanized vials with 150 µl of 2 *M* hydrochloric acid in methanol at 60 °C for 10 min and immediately evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 50 µl of acetonitrile and 100 µl of PFPA, heated at 60 °C for 20 min and evaporated to dryness in a gentle stream of nitrogen after cooling to room temperature. The residue was recovered in 50 µl of methylene chloride and 1–2 µl were injected into the GC–MS system.

Gas chromatography–mass spectrometry

A VG micromass 16 F magnet-sector mass spectrometer (Vacuum Generator Analytical, Manchester, U.K.) was used both for recording full mass spectra and for SIM. Chromatographic separation of the derivatives was achieved on a DB-1701 fused-silica capillary column 30 m × 0.25 mm, film thickness 0.25 µm, J&W Scientific, Rancho Cordova, CA., U.S.A.) using a helium flow-rate of 1.0 ml/min. The samples were injected via a solvent-free injector (dropping needle type [11], Ray Allen Assoc., Boulder, CO, U.S.A.). The injector temperature was set to 220 °C, the transfer line to 250 °C. The end of the column was directly inserted into the mass spectrometer source, which was operated at 70 eV and a temperature of 180 °C. The GC oven was kept at 130 °C for 2 min following the injection

and heated at a rate of 8°C/min to a final temperature of 180°C which was held for 10 min. Methane and isobutane were used as reagent gases for positive-ion chemical ionization (PICI).

Electron capture NICI mass spectra and SIM were carried out on a Nermag R10-10C quadrupole mass spectrometer (Nermag Instruments, Rueil-Malmaison, France) equipped with a DB-1 fused-silica capillary column (20 m×0.25 mm, film thickness 0.25 μm, J&W Scientific) using a helium flow-rate of 60 cm/s. The source temperature was 180°C and the transfer line was kept at 300°C. Methane was used as the PICI moderating gas at a source pressure of 13 Pa. The GC oven was initially held at 100°C for 2 min after splitless injection and then raised to 180°C at a rate of 5°C/min. After completion of the analysis, the column was heated to 300°C at a rate of 30°C/min.

Sample concentrations were quantified by isotope dilution calibration curves using the peak-height ratio method (electron ionization and PICI) or peak-area ratio method (NICI) of deuterated and unlabeled catecholamines.

High-performance liquid chromatography–electrochemical detection

The HPLC–ED system consisted of a Shimadzu LC-6A solvent delivery module (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.), an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and an ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.). The catecholamines were separated on an Ultrasphere-ODS 5-μm column (250 mm×4.6 mm, Beckman Instruments, San Ramon, CA, U.S.A.). The mobile phase consisted of 0.15 *M* monochloroacetic acid adjusted to pH 2.9 which contained 0.7 m*M* octanesulfonic acid sodium salt, 0.5 m*M* EDTA and 4.5% acetonitrile (v/v). The flow-rate was 1.0 ml/min. Buffers were degassed by filtration (0.45 μm). The applied potential was +600 mV vs. Ag/AgCl. Concentrations were calculated from peak-height ratios of catecholamines and DHBA.

Statistics

Values are expressed as means ± S.D. Statistical comparison of the data was performed by analysis of variance followed by unplanned multiple comparison of the means. A probability of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The brown residue, which quickly built up on the injector tip of the gas chromatograph, was thought to consist of non-volatile macromolecules that were non-specifically adsorbed on alumina. Therefore, an additional clean-up step on Sephadex G-10 was introduced to remove these high-molecular-mass impurities. Sephadex has been used to isolate catecholamines for HPLC analysis from tissue [12,13] and in combination with alumina or ion-exchange resins from body fluids [14]. However, this procedure requires perchloric acid, which led to decomposition of the catecholamines when derivatization was attempted. Furthermore, the large elution volumes used are unsuitable for the detection of very small amounts of compounds [15]. Temperature control of the alumina work-up step in com-

bination with the handling of large volumes of eluents made this method laborious and very time-consuming [14]. Thus, the procedure was modified employing alumina adsorption as a pre-concentration step followed by purification on Sephadex. A smaller column packing further reduced the elution volume. 0.1 *M* Hydrochloric acid was used as eluent. All steps can be carried out at room temperature, which further simplifies the method. Up to twenty samples were processed in less than 2 h. The four basic catecholamines, epinephrine, norepinephrine, dopamine and epinine, eluted with a volume of 1 ml. The recovery of the four catecholamines from standard mixtures, prepared in 0.1 *M* hydrochloric acid, from Sephadex G-10 was >95% as determined by HPLC–ED. Acidic metabolites of catecholamines, such as DOPAC, were separated from the basic compounds by Sephadex and were collected in the subsequent milliliter of eluent [12,13]. This fraction was not actually included in the SIM GC–MS assay.

The overall recovery for both alumina and Sephadex purification of epinephrine and epinine and the results of quantification of 25 pmol of both catecholamines added to cerebellar tissue samples are summarized in Table I. Norepinephrine and dopamine, which can be determined in the same derivatized extract, were not included in these experiments because they are present in all areas of the brain. Compared to alumina extraction as the only purification method, additional Sephadex work-up resulted in 2.5- and 7-fold increase of the peak height of epinephrine-α,α,β-d_3 isolated from hypothalamus and brainstem, respectively (Table II). Furthermore, no peak broadening or contaminant accumulation on the injector tip was observed for these samples. Generally, a slightly lower peak height was found for catecholamines isolated from brainstem compared to hypothalamus. This might be due to the fact that more brain tissue was used for these samples, which may result in a lower recovery.

Derivatization of the catecholamines was achieved by treatment with 2 *M* hydrochloric acid in anhydrous methanol prior to the reaction with PFPA, yielding the β-methoxy-tris(pentafluoropropionate) derivatives of the β-hydroxylated compounds epinephrine and norepinephrine as well as the pentafluoropropionates of dopamine and epinine. All derivatives are formed in quantitative yield

TABLE I

EXTRACTION EFFICIENCY AND GC–MS QUANTIFICATION

A 25-pmol amount of each catecholamine, added to minced cerebellar tissue, was extracted on alumina and further purified on Sephadex G-10. The total recovery was determined by HPLC–ED. The quantification by SIM GC–MS was determined by electron-impact GC–MS after sample extraction and derivatization of 25 pmol of unlabeled catecholamine and deuterated internal standard added to minced cerebellum. For chromatographic conditions see the Experimental section. The values are expressed as mean ± S.D. of three experiments. Each sample was analyzed twice.

Catecholamine	Recovery (%)	Quantification (pmol)
DHBA	78.2 ± 5.3	–
Epinephrine	76.7 ± 4.1	24.49 ± 0.21
Epinine	77.6 ± 2.1	25.07 ± 1.88

TABLE II

EFFECT OF SEPHADEX G-10 PURIFICATION STEP ON THE PEAK HEIGHT

A 25-pmol amount of epinephrine-α,α,β-d_3, added to minced hypothalamus and brainstem, was extracted with and without the Sephadex G-10 purification step and compared to the peak height of a standard. The most abundant ion of the mass spectrum, *m/z* 446, was recorded For derivatization and chromatographic conditions see the Experimental section. The values are expressed as mean ± S.D

Tissue	*n*	Sephadex purification	Peak height	Percentage relative to standard
Standard	8	–	7260 ± 987	100.0
Hypothalamus	7	No	2048 ± 373	28.2
Hypothalamus	7	Yes	5215 ± 1090	71.8
Brainstem	3	No	627 ± 136	8.6
Brainstem	3	Yes	4323 ± 704	59.5

and have excellent GC and MS properties. The short-term acid treatment led to only an insignificant loss (about 5%) of deuterium bound to aromatic rings. This is in accordance with other researchers who did not observe exchange under acidic conditions at room temperature after longer periods of time [16,17].

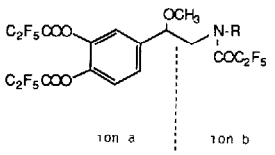
The mass spectra of the β-methoxy-tris(pentafluoropropionate) derivatives of epinephrine and norepinephrine under different ionization conditions are summarized in Table III. Characteristic fragments at high *m/z* values with sufficient intensity for SIM GC–MS are observed under all conditions. The base peak *m/z* 445, resulting from the dominant α-cleavage in the electron ionization mode, represents the aromatic moiety of the molecule (ion a). In contrast, the mass spectra of the frequently prepared tetra(pentafluoropropionate) derivatives of norepinephrine and epinephrine, which are obtained by a one-step reaction with PFPA, display only a single intense ion at *m/z* 176 and *m/z* 190, respectively. These ions represent the uncharacteristic side-chain fragment (ion b) [2,6,7,18]. Considering the complexity of a biological matrix such as brain tissue, derivatives yielding fragments at high masses increase the specificity of the analysis. Chemical ionization of the β-methoxy-tris(pentafluoropropionates) generated an intense ion due to the loss of a CH_3O fragment which eventually became the most abundant ion when isobutane was used as the reagent gas. The electron capture NICI mass spectra are characterized by the loss of a C_2F_5CO moiety. The NICI spectra of epinephrine and norepinephrine display a single intense ion at *m/z* 488 and *m/z* 474, respectively. The spectra of dopamine and epinine were in accordance with previously published data [19–21].

Combined with the Sephadex G-10 purification step, recording the base peaks of the respective mass spectra resulted in a very sensitive SIM GC–MS assay. Electron ionization GC–MS had about the same sensitivity as HPLC–ED. The detection limits for both methods were found to be about 30–50 fmol. Methane or isobutane chemical ionization slightly decreased the sensitivity due to an enhanced background noise level resulting from the reagent gas. The peak height is further reduced by the fact that the fragment *m/z* 604, monitored in the case of

TABLE III

CHARACTERISTIC IONS AND RELATIVE INTENSITIES IN THE MASS SPECTRA OF THE β-METHOXY-TRIS(PENTAFLUOROPROPIONATE) DERIVATIVES OF EPINEPHRINE AND NOREPINEPHRINE

A 100-μg amount of the catecholamine was derivatized with 2 *M* hydrochloric acid in methanol followed by reaction with PFPA as described in the Experimental section. Ionization conditions applied were: electron impact (EI), positive-ion chemical ionization (PICI), methane negative-ion chemical ionization (NICI). Relative intensities are given in parentheses.



	$[M+1]^+$	M^+	$M^+ - OCH_3$	$M^- - COC_2F_5$	Ion a	Ion b
Epinephrine						
EI	–	635 (<0.5)	604 (<0.5)	–	445 (100)	190 (72)
Methane PICI	636 (13)	–	604 (90)	–	445 (100)	190 (25)
Isobutane PICI	636 (6)	–	604 (100)	–	445 (64)	190 (10)
NICI	–	635 (<0.5)*	–	488 (100)	–	–
Norepinephrine						
EI	–	621 (<0 5)	590 (<0.5)	–	445 (100)	176 (60)
Methane PICI	622 (5)	–	590 (87)	–	445 (100)	176 (16)
Isobutane PICI	622 (10)	–	590 (100)	–	445 (62)	176 (12)
NICI	–	621 (<0.5)*	–	474 (100)	–	–

*M^- ion.

epinephrine, represents a lower percentage of the total ions formed compared to the base peak of the electron ionization spectrum. In contrast, NICI proved to be extremely sensitive. A 3.5-pg amount of injected pentafluoropropionate derivative of epinine could easily be detected (Fig. 1C). The signal-to-noise ratio (7:1) suggested that even 1 pg of derivative (approximately 1.5 fmol) should be measureable for a detection limit defined as a signal-to-noise ratio of 2:1. This corresponds to a 25-fold increase in detection limits compared to electron ionization GC–MS or HPLC–ED. When used to test samples from rat hypothalamus and brainstem (Fig. 1D), this method allowed the detection of endogenous epinine. These levels were only detectable after inhibition of monoamine oxidase by pargyline, which is known to increase tissue levels of biogenic amines [22]. Because the amount of epinine was below the lowest concentration used for the calibration curve, reliable quantification could not be achieved. The concentration was estimated to be 0.01–0.04 pmol/mg of protein. Epinine has been found in the superior cervical ganglion of the newborn rat [20], but was previously not described in brain tissue. Currently, it cannot be excluded that the epinine found is an artifact due to pargyline incubation, although hardly detectable levels were also present

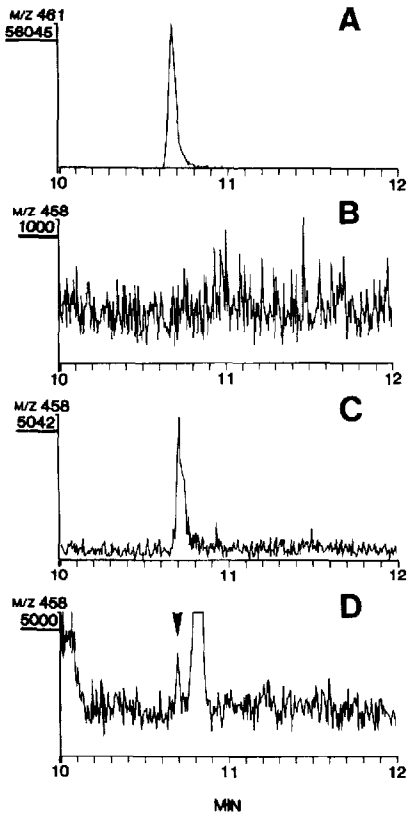


Fig. 1. NICI ion currents of unlabeled epinine and epinine-d_3. (A) Epinine-d_3 (*m/z* 461) (34.5 pg injected); (B) unlabeled epinine (*m/z* 458) channel recorded from the injection of 34.5 pg epinine-d_3; (C) epinine (3.4 pg injected); (D) hypothalamus sample, the arrow head indicates possible endogenous epinine. Samples were prepared and derivatized as described in the Experimental section. The compounds were separated on a 20-m DB-1 fused-silica capillary column kept initially at 100 °C and increased to 180 °C at a rate of 5 °C/min beginning 2 min after the injection.

in a sample of unincubated tissue. Co-elution of a decomposition product of pargyline with identical mass seems very unlikely. On the other hand, epinine levels may be the result of the accumulation of other biogenic amines caused by monoamine oxidase inhibition. This might be interesting for the pharmacological use of monoamine oxidase inhibitors for the treatment of depression and parkinsonism [23].

The practical application of the GC–MS assay was evaluated by comparison of electron ionization SIM GC–MS with HPLC–ED using the dopamine-β-hydroxylase-mediated conversion of epinine-d_6 to epinephrine-d_6 by rat hypothalamus and brainstem in vitro. This reaction was chosen because epinine has previously not been found in brain and epinephrine is endogenously present in these tissues [18,24]. GC–MS has the advantage of being able to differentiate between endogenous unlabeled compound and species derived from a stable isotope-labeled precursor. Fig. 2 shows the results obtained from incubation of minced hypothalamic or brainstem tissue with 1 μM epinine-d_6. The levels of endogenous unlabeled

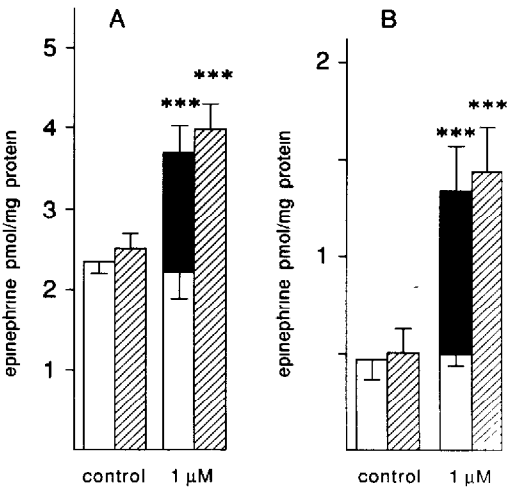


Fig. 2. Conversion of epinine-d_6 to epinephrine-d_6 by rat hypothalamus (A) and brainstem (B) in vitro. Samples were incubated for 20 min at 37°C and extracted and derivatized as described in the Experimental section. (□) Endogenous epinephrine determined by SIM GC–MS (m/z 445); (■) epinephrine-d_6 formed from epinine-d_6 determined by SIM GC–MS (m/z 448); (▨) total epinephrine analyzed by HPLC–ED. The values are expressed as mean ± S.D of five experiments; each sample was analyzed twice; (***) $P < 0.001$ versus control.

epinephrine (open bars) did not change during the experiment while epinephrine-d_6 (solid bars) appeared upon incubation with epinine-d_6. The total epinephrine as sum of labeled and unlabeled compound is in excellent agreement with the values obtained by HPLC–ED (hatched bars). These results further confirm our observation that acid-catalyzed deuterium–hydrogen exchange of deuterium bound to aromatic rings was neglectible during short-term heating with 2 *M* hydrochloric acid in methanol.

In conclusion, the described assay proved to be very sensitive and specific. This appears to be partly due to the separation and purification of the samples in the Sephadex clean-up and partly to the high-mass ions used to measure the biogenic amines. It is suitable for the detection of very small amounts of endogenous biogenic amines as well as for the investigation of biosynthetic pathways using stable isotope-labeled precursors.

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