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Liquid Chromatographic Assay for Cerebrospinal Fluid Normetanephrine

Thomas H. Marshall, Kenneth A. Jacobson*, Kenneth L. Kirk*, and Markku Linnoila[°]

Laboratory of Clinical Studies, DICBR, National Institute on Alcohol Abuse and Alcoholism

Summary

A method for quantitation of normetanephrine in human cerebrospinal fluid is described. An amine-specific reagent, sulfosuccinimidyl propionate, is used to obtain the lipid soluble N-propionyl derivative of normetanephrine, which can be separated and quantitated in presence of other biogenic amines by liquid chromatography with electrochemical detection. The method is reproducible, linear, and precise at the relatively low concentrations of unconjugated normetanephrine occurring in human cerebrospinal fluid. Hospitalized, drug-free, alcoholic patients were found to have cerebrospinal fluid unconjugated normetanephrine concentrations in the 0.5-1.5 nanomolar range. The practical limit of sensitivity for the method is about 0.025 pmole per ml of CSF.

The measurement of biogenic amines and their metabolites is clinically important in disorders involving the sympathetic nervous system. For instance, the quantitation of normetanephrine, a metabolite of the neurotransmitter norepinephrine, is of interest in patients with hypertension and catecholamine excreting tumors such as pheochromocytoma and neuroblastoma (1-7).

Additional impetus to measure normetanephrine stems from the catecholamine hypothesis of affective disorders, which states that depressions result from a relative deficit of intrasynaptic norepinephrine in the central nervous system (8). Norepinephrine is metabolized via two pathways: oxidation to 3-methoxy-4-hydroxyphenyl glycol and vanillylmandelic acid (VMA), or non-oxidatively by o-methylation to normetanephrine. Norepinephrine metabolism can be studied in humans by measuring the concentrations of these metabolites and the parent amine in various body fluids. Possible insight can be gained into the mechanism of drug action during treatment of depressive illness by measuring normetanephrine in relation to norepinephrine turnover and metabolic profiles (9, 10). We report a liquid chromatographic method for quantitation of normetanephrine in human cerebrospinal fluid (CSF). A key feature of the method is the acylation of normetanephrine to produce a lipid-soluble compound which is amenable to a high yield concentration step during the extraction procedure.

[°]Address for correspondence: NIAAA, Building 10, Room 3B19, 9000 Rockville Pike, Bethesda, MD 20892.

*laboratory of Chemistry, National Institute on Arthritis, Diabetes, and Digestive and Kidney Diseases, Bethesda, MD, U.S.A

Material and Methods

Sulfosuccinimidyl Propionate, Sodium Salt, 2b

The derivatizing reagent was synthesized as follows: N-hydroxysulfosuccinimide (1.0g, 4.2 mmol), Pierce Chemical, Rockford, IL was suspended in 10 ml of dimethylformamide (DMF) and treated with propionic anhydride (0.9 ml, 7 mmol). After stirring overnight the solids were nearly completely dissolved. Addition of ethyl acetate (50 ml) and petroleum ether (30 ml) precipitated the product (1.16g, 100% yield) which was dried in a vacuum oven at 50°C. An analytical sample (mp 239-242°C) was recrystallized from DMF/ether, calculated ($C_7H_8NO_7NaS$) 30.78% C, 2.95% H, 5.13% N; found 30.67% C, 3.00% H, 5.07% N. NMR [$(CD_3)_2SO$]: resonances in ppm were 3.95 (1H, m, $CHSO_3$) 2.88 and 2.82 (2H, m, CH_2CON), 2.68 (2H, q, CH_2COO) 1.14 (3H, t, CH_3). IR showed a strong ester band at 1750 cm^{-1} .

N-Propionyl Normetanephine 3a

External standard was synthesized as follows: D,L-Normetanephine hydrochloride (50 mg, 0.23 mmol) and sulfosuccinimidyl propionate (40 mg, 0.15 mmol) were dissolved in 1 ml of water. Saturated sodium bicarbonate (1 ml) and 10% sodium carbonate (0.1 ml) were added (gas evolution). After one half hour the solution was extracted six times with ethyl acetate. The combined organic layer was dried with $MgSO_4$ and evaporated leaving a clear glass, which was homogenous by thin layer chromatography (32 mg, 91% yield). Analysis ($C_{12}H_{17}NO_4$): calculated 60.24% C, 7.16% H, 5.85% N; found 59.98% C, 7.24% H, 5.77% N. Accurate mass of product after water elimination: calculated 221.1052; found 221.1054.

N-Propionyl 3-ethoxy-4-hydroxyphenylethanolamine, 3b, was prepared in a similar manner from 3-ethoxy-4-hydroxyphenylethanolamine (EHPEA) neutral oxalate, 1b (11); Accurate mass: calculated ($C_{13}H_{19}NO_4$) 253.1314; found 253.1310.

Commercial Reagents

D,L-Normetanephine hydrochloride (NMN) was from Sigma Chemical Co, St. Louis, MO. Sodium chloride, potassium chloride, acetone, disodium ethylene diamine tetracetate and sodium bicarbonate were from J.T. Baker Chemical Company, Phillipburg, NJ. Monobasic and dibasic sodium phosphate and glacial acetic acid were from Mallinckrodt Chemical Works, St. Louis, MO; magnesium chloride was from Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH; HPLC grade ethyl acetate and calcium chloride dihydrate were from Fisher Scientific, Fairlawn, NJ. Deionized water from a Millipore deionizer was used throughout.

Chromatography

A Waters Associates, Milford, MA, 6000 A pump and U6K injector were used with a Coulchem 5100 A coulometric detector from Environmental Sciences Associates, Bedford, MA, and a Phenomenex, Rancho Palos Verdes, CA, "Ultrex" C 18, 5 μ m particle size, 150 $\times$ 4.6 mm column. A mobile phase consisting of 6.5% acetone in 0.08 M sodium acetate, acetic acid, pH 4.6, containing 0.01% EDTA was prepared fresh daily, filtered through a 0.45 Millipore filter, degassed under vacuum and pumped at a flow rate of 1.3 to 1.5 ml/min.

All chromatography was performed at room temperature. The detector setting was 0.35 volts in the oxidative mode. A Kipp and Zonen double channel chart recorder was used to produce the chromatograms. Peaks were identified by retention times using authentic standards and by adding normetanephrine to artificial and human CSF. At the beginning and end of each day's run standards with known concentrations of the N-propionyl nor-metanephrine derivative were injected to check the chromatographic conditions.

Derivatization and Extraction

One ml aliquots of artificial or human cerebrospinal fluid (CSF) were pipetted into silanized, screw cap culture tubes followed by addition of 100 μ l of EHPEA to give a final concentration of 1 μ mole/ml EHPEA. To this was added 25 μ l of freshly dissolved 0.4 M sodium sulfo N-succinimidyl propionate and 100 μ l of saturated K_2CO_3 followed by vortexing and capping. After 10 minutes at room temperature 100 μ l of saturated NaCl, which had been filtered through a 0.45 μ m filter, was added followed by vortexing. The derivative was extracted twice by vortexing with 3.0 ml of ethyl acetate for 15 seconds followed by a 5 minute centrifugation step to separate the layers. The combined organic extracts were evaporated with heating to dryness on a Speed Vac, Savant, Hicksville, NY, evaporator. The residue was redissolved in 100 μ l of water and injected.

Precision was determined by preparing series of 6 ml aliquots of artificial CSF containing 3 known concentrations of normetanephrine and salts in the following amounts: NaH_2PO_4 0.5 mM, Na_2HPO_4 0.25 mM, $MgCl_2$ 0.4 mM, $CaCl_2$ 0.65 mM, KCl 3.0 mM, NaCl 128 mM and $NaHCO_3$ 25 mM. The salts were dissolved on a magnetic stirrer for 15 minutes and the clear solution was filtered through a Millipore filter. Normetanephrine was added and individual tubes stored at -20° . On each of five days, one tube of each concentration was thawed and quantified separately in five 1.0 ml aliquots (a total of 15 assays).

Linearity in Human CSF

A standard curve from a pool of human CSF spiked with normetanephrine and EHPEA is shown in Fig. 3. The ratio of the heights of the normetanephrine peak to the EHPEA peak was used to determine the normetanephrine concentration.

Human CSF was collected by lumbar puncture from alcoholic patients in the lateral decubitus position after an overnight bed rest. The patients were housed on a locked research ward. They had been free of medications and alcohol for a minimum of 21 days prior to sampling and were maintained on a closely supervised low monoamine diet (12). The samples were stored tightly capped at -60° .

Results

Following our previously described acylation procedure (13, 14), the primary amino group of normetanephrine was acylated selectively with a large excess of either succinimidyl propionate, 2a, or sodium sulfosuccinimidyl propionate, 2b (Fig. 1). The reaction with 2a required an additional quenching step which consisted of the addition of an excess of a highly polar amine such as glycine, to prevent extraction of any unreacted ester into the

organic phase. For this reason, the water soluble sulfoanalog, 2b, proved to be the derivatizing reagent of choice.

The product of acylation, N-propionyl normetanephrine, was prepared for HPLC comparison as a pure amorphous solid, which gave the correct elemental and spectroscopic analysis. 3-Ethoxy-4-hydroxyphenylethanolamine (EHPEA 1b) Fig. 1, was prepared as the oxalate salt, (11) and used as an internal standard, of comparable chemical reactivity, polarity, and electroactive properties to normetanephrine. The N-propionyl derivatives of both normetanephrine and EHPEA were efficiently extracted into ethyl acetate (Table I). After extraction, the organic layer was separated and evaporated, providing a means for increasing sensitivity of detection by concentration and exclusion of polar interfering substances.

The derivatized NMN and EHPEA peaks have retention times of around 5 and 10 minutes respectively (Fig. 2). The peak heights are individually proportional to added NMN and EHPEA concentrations and disappear if sulfosuccinimidyl propionate is omitted from the derivatizing mixture. The reaction is equally efficient with the analyte and internal standard when a large excess of derivatizing agent is used. Reducing sulfosuccinimidyl propionate concentration by a factor of 16 decreased the peak heights by 45%, but left their ratio unchanged (Table I). The efficiencies of extracting normetanephrine and EHPEA derivatives into ethyl acetate from water and sodium chloride solution are nearly 100% (Table II).

Both the linearity of the detector response and precision of the assay are good (Table III, Fig. 4). The within run coefficient of variation (%CV) ranged from 3.22 to 10.5%. Other biogenic amines which might interfere are clearly resolved from normetanephrine under our chromatographic conditions. The N-propionyl derivatives of dopamine, epinephrine, norepinephrine and octopamine are separated from one another and from normetanephrine (Fig. 6). Serotonin and N-acetyl serotonin have even longer retention times (14). A group of drug free, hospitalized alcoholic patients had levels of CSF normetanephrine in the 0.5 - 1.5 pmol/mL range (Table IV).

Discussion

Our derivatization method is based on a new specific acylating reagent, sulfo N-succinimidyl propionate. This water soluble reagent reacts specifically with amines in the presence of aromatic hydroxyl groups to produce a lipid soluble derivative while preserving the electrochemical activity of the hydroxyl group (13). Previously we used a lipid soluble agent, N-succinimidyl propionate, to derivatize serotonin (14). It was necessary to add glycine as a [quenching] step to prevent the unreacted derivatizing reagent from contaminating the derivative. Thus, the use of the water soluble sulfo N-succinimidyl propionate provides a practical advance. We have used the normetanephrine homologue, EHPEA as an internal standard. It is similar enough to react with the derivatizing agent and extract into ethyl acetate with efficiency nearly equal to normetanephrine, and yet different enough to be well separated in the chromatography.

Most previous assays for normetanephrine were applied to either urine or plasma. For instance an HPLC-EC method for urinary normetanephrine was not sufficiently sensitive to quantitate CSF normetanephrine (15). A highly sensitive radioenzymatic method based on converting normetanephrine to [³H] metanephrine in the presence of [³H] S-adenosyl methionine and phenylethanol-amine N-methyl transferase (16) has been used for human CSF (5). The values reported (5), 126 ± 14 and 70 ± 14 ng/L for hypertensive and normotensive individuals are somewhat lower than the mean (150 ± 65 ng/L) but close to the range (78 - 264 ng/L) we report here for 13 hospitalized alcoholic patients (Table IV). The radioenzymatic method has a 10% cross reactivity with octopamine (17). Our method avoids this problem because the chromatographic separation of the normetanephrine and octopamine derivatives is complete (Fig. 6). A mass fragmentographic determination of unconjugated normetanephrine in the plasma of three healthy individuals yielded a concentration of 264 ng/L (3), a value approximately twice the mean but within the range of the CSF samples reported here.

In conclusion, we have developed a rapid and reliable liquid chromatographic method for quantitation of unconjugated normetanephrine. The precision and linearity of this method have been established in the concentration range for unconjugated normetanephrine in human CSF. The method allows eighteen samples and a standard curve to be analyzed in an average working day.

References

1. Wolf RL, Mendlowitz M, Roboz J, Gitlow SE. New England J of Med. 1965; 273:1459–1463. [PubMed: 5852903]
2. Crout JR. Anesthesiology. 1968; 29:661–669. [PubMed: 4874151]
3. Wang MT, Yoshioka M, Imai K, Tamura Z. Clin Chim Acta. 1975; 63:21–27. [PubMed: 1175284]
4. Kobayashi K, Dequattro V, Bornheimer J, Kolloch R, Miano L. Life Sci. 1980; 26:1821–1826. [PubMed: 7392815]
5. Dequattro V, Sullivan P, Minagawa R, Koinn I, Bornheimer J, Foti A, Barndt R. Fed Proceedings. 1984; 43:47–51. [PubMed: 6690337]
6. Foti A, Adachi M, Dequattro V. J Clin Endocrinol Metabolism. 1982; 55:8185.
7. Kobayashi K, Kolloch R, Dequattro V, Miano L. Clinical Sci. 1979; 57:173–176. [PubMed: 477254]
8. Koslow SH, Maas JW, Bowden CL, Davis JM, Hanin I, Javaid J. Arch Gen Psychiatry. 1983; 40:999–1010. [PubMed: 6193764]
9. Linnoila M, Karoum F, Calil HM, Kopin IJ, Potter WZ. Arch Gen Psychiatry. 1982; 39:1025–1028. [PubMed: 6214234]
10. Potter WZ, Karoum F, Linnoila M. Prog Neuropsychopharmacol Biol Psychiatry. 1984; 8:153–161. [PubMed: 6233636]
11. Brown RT, Kirk KL, Oliver J. J of Liquid Chromatog. 1986 in press.
12. Muscettola G, Wehr T, Goodwin FK. Am J Psychiatry. 1977; 134:914–916. [PubMed: 879357]
13. Jacobson KA, Marshall TH, Mine K, Kirk KL, Linnoila M. FEBS Let. 1985; 188:307–311. [PubMed: 4029392]
14. Linnoila M, Jacobson KA, Marshall TH, Miller TL, Kirk KL. Life Sci. 1986; 38:687–694. [PubMed: 3951324]
15. Shoup RE, Kissinger PT. Clin Chem. 1977; 23:1268–1274. [PubMed: 872373]
16. Vlachakis ND, Dequattro V. Biochem Med. 1978; 20:107–114. [PubMed: 718674]
17. Kobayashi K, Foti A, Dequattro V, Kolloch R, Miano L. Clin Chim Acta. 1980; 107:163–173. [PubMed: 6777088]

Fig. 1.
Derivatization of normetanephrine and 3-methoxy-4-hydroxy-phenylethanolamine.

Fig. 2.

Chromatogram of derivatized human CSF pool spiked with NMN using EHPEA as internal standard. A pool of human CSF was spiked with NMN (0.75 pmol/mL) and EHPEA (1.0 pmol/mL) and derivatized, extracted and injected into the HPLC. The column was an Ultrex CI8, 5 μ , 15 $\times$ 0.46 cm. The mobile phase; 6.5% acetone, 0.08 M Na acetate, acetic acid, pH 4.6. In the absence of spiking the NMN peak was one third as high as shown.

Fig. 3. Human pooled CSF spiked with various amounts of NMN ($2.5 \times 10^{-8}\text{M}$) and treated as in Fig. 1.

Fig. 4. Between run mean $\pm$ S.D reproducibility of the assay from artificial CSF spiked with normetanephrine.

Fig. 5.
A CSF sample of an alcoholic patient spiked with EHPEA and treated as in Fig. 1.

Fig. 6.

A chromatogram showing the relative retention times of normetanepheive (NE), octopamine (OA), normetanephine (NMN), epinephrine (E) and dopamine (DA) in artificial CSF.

Table I
Effect of Derivatization Reagent Concentration on NMN and EHPEA Peak Heights

Final Acylating Reagent Cone.		Percent Maximum Peak Height		
Reagent Cone.		NMN [*]	EHPEA [†]	Ratio
mM				
8		100	100	
4		91	96	0.95
2		85	89	0.96
1		66	74	0.89
0.5		55	58	0.95
0.25		28	32	0.88
0.125		2	19	0.11

^{*} NMN = normetanephrine,

[†] EHPEA = 3-Ethoxy-4-hydroxyphenylethanolamine.

Table II
Efficiency of Extraction of NMN and EHPEA into Ethyl Acetate Peak height mm

	<u>NMN</u>	<u>EHPEA</u>	<u>Ratio</u>
Pre Extraction N = 5			
Means $\pm$ SD	44 $\pm$ 1.2	74.6 $\pm$ 2.3	0.59 $\pm$.017
% Yield	100.0	100.0	100.0
Extraction from Water N = 4			
Mean $\pm$ SD	43.5 $\pm$ 1.9	66.5 $\pm$ 1.7	0.65 $\pm$.015
% Yield	98.8	89.1	110.1
Extraction from 8% Saturated NaCl N = 3			
Means $\pm$ SD	43.6 $\pm$ 2.3	69 $\pm$ 1.4	0.63 $\pm$.04
% Yield	99.0	92.5	106.8

Table III
Within Run Reproducibility Measuring Normetanephine Using EHPEA[†] as Internal Standard

NMN pMoles/mt	Peak Ratio Mean $\pm$ S.D.		
0.1	0.1947 $\pm$ 0.0212	% CV [*] = 10.9	N = 18
0.4	0.8267 $\pm$.0652	% CV = 7.90	N = 21
1.0	2.016 $\pm$ 0.1772	% CV = 8.79	N = 23

[†] concentration of EHPEA was 1.0 pMoles/mL.

$$^* \% CV \frac{S.D.}{Mean} \times 100$$

Table IV
Levels of Normetanephrine in CSF of Hospitalized, Abstinent Alcoholic Patients

	pmol/mL	
1	1.39	
2	0.583	
3	1.44	
4	1.22	
5	0.495	
6	0.597	
7	0.950	Mean $\pm$ S.D. 0.846 $\pm$ 0.370
8	0.550	
9	0.598	
10	0.897	
11	0.442	
12	1.285	
13	0.555	