Journal of Chromatography, 108 (1975) 43–52

CHROM. 8173

QUANTITATIVE DETERMINATION OF EPINEPHRINE AND NOREPINEPHRINE IN THE PICOGRAM RANGE BY FLAME IONIZATION GAS–LIQUID CHROMATOGRAPHY*

H. G. LOVELADY and L. L. FOSTER

Biodynamics Branch, Environmental Sciences Division, USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, United States Air Force, Brooks AFB, Texas 78235 (U.S.A.)

(Received November 29th, 1974)

SUMMARY

A gas–liquid chromatographic method has been developed using the hydrogen flame detector to determine epinephrine (E) and norepinephrine (NE) in blood plasma, red blood cells, serum, and urine. The chromatographic method presents several advantages over other existing techniques. The derivatives enable separation of E and NE and are stable at room temperature with no signs of decomposition. The detection limit for the catecholamines with the hydrogen detector was approximately 0.1 pg. The catecholamines can be determined simultaneously from the same gas–liquid chromatogram. Purification of the catecholamines using the conventional procedure of chromatographing on alumina has been eliminated. With this gas chromatographic method, no by-products are formed that interfere with E and NE determinations. Dopamine, which constitutes the major source of interference in the commonly used fluorometric methods, does not interfere with the E and NE determinations. Norepinephrine and epinephrine values for several physiological fluids are given with the analysis expanded to include red blood cells, the contents of which have not been previously reported.

INTRODUCTION

Using gas chromatographic techniques to separate biologic amines, including catecholamines, requires preparing suitable derivatives. One major problem in developing a gas–liquid chromatography (GLC) procedure for the polyfunctional compounds such as epinephrine (E) and norepinephrine (NE) is that multiple products may be formed when converting to derivatives.

Capella and Horning[1] separated the catecholamines by using a 10% F-60 column with temperature programming. Sen and McGeer[2] obtained trimethylsilyl

* The animals involved in this study were maintained and used in accordance with the Animal Welfare Act of 1970 and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences–National Research Council.

(TMS) ether derivatives from E and NE, but found that the primary and N-methyl pairs of derivatives were not separable with ordinary columns. Brochmann-Hanssen and Svendsen[3] used acetone to study the condensation products of ephedrine and related amines. Linstedt[4] showed that TMS ethers could be obtained from human biologic amines; the structures were studied only by GLC techniques. To find a procedure which will lead to a single product from each component of an amine mixture containing catecholamines, many reaction conditions must be studied. Moffat and Horning[5] stated that in a derivatizing condition, E is converted to two isomeric substituted tetrahydroisoquinolines which interfere with the NE peak on a 5% SE-30 column. They also indicated that the electron capture detector is approximately 2000 times as sensitive as the flame ionization detector. Horning *et al.*[6] separated the catecholamines by temperature programming with 5% OV-1 (a methylsiloxane polymer) and 5% OV-17 (a phenylmethylsiloxane polymer) columns, but the time required to complete a reaction using NE as a model compound was 5 h in acetonitrile and 50 h in pyridine. They also found that N, O-bis(trimethylsilyl)acetamide (BSA) or BSA–trimethylsilylchlorosilane (TMCS) silylated catecholamine primary amino groups twice, and the secondary groups slowly or not at all. Horning *et al.*[7] studied the silylation of catecholamine hydroxyls with trimethylsilylimidazole (TSIM), followed by N-acylation using N-acetyl- or N-heptafluorobutyrylimidazole. On GLC of the reaction mixtures (flame ionization detector), the acetyl derivatives showed longer retention time and trailed slightly on SE-30, OV-1, and OV-17. Kawai and Tamura[8,9] used hexamethyldisilazane (HMDS) in dimethylsulfoxide and found it to be a superior solvent to that of dimethylformamide[1]. Kawai *et al.*[10] satisfactorily separated the catecholamine and related compounds through trimethylsilylation with HMDS, using pyridine as a solvent, followed by condensation with 2-pentanone. Later, pyridine was found unsuitable to quantitatively determine such extremely unstable catecholamines, since it required a longer time at a higher temperature for complete trimethylsilylation. Kawai and Tamura[11] stated that because of the limited sensitivity of the hydrogen flame ionization detector, the method is still inapplicable to materials of low catecholamine concentration such as urine. They[11] also reported a method using trifluoroacetyl derivatives with an electron capture detector, and indicated that this method seemed superior to the fluorometric method with regard to E and NE determination.

Imai *et al.*[12] reported a method for determining catecholamines in rat tissues and serum, but they only had a 50% recovery.

Wong *et al.*[13] used the electron capture detector to measure the catecholamines NE, E, and dopamine in normal human urine as their pentafluoropropionyl derivatives. When they used alumina, their recovery of amines added to urine varied from 10–50%. Sourkes and Drujan[14] in their study reported that the recovery is highly variable and may be as low as 15%. Imai *et al.*[15] reported a method, using an electron capture detector, to determine catecholamines in plasma of normal persons and patients with hypertension; however, the content of E was below 0.1 ng/ml of plasma and could not be determined.

Gas chromatography has several advantages over fluorometry. Each peak reliably indicates the amount of catecholamines without interference by other coexisting components, whereas fluorometry often gives unreliable information since E and NE amounts are calculated by simultaneous equations in which dopamine interferes. We have developed a GLC method to separate and quantitate E and NE

using the dual hydrogen flame detector. Separations were carried out with a 7% DC-11 on 80–100 mesh Gas-Chrom P column, under isothermal conditions.

EXPERIMENTAL

Gas–liquid chromatography

A Beckman GC-5 chromatograph with a dual hydrogen flame detector was used. The stainless-steel columns (6 ft. × 1/8 in. O.D.) were packed in our laboratory with 7% DC-11 on 80–100 mesh Gas-Chrom P (Applied Sciences Labs., State College, Pa., U.S.A.), and conditioned for 48 h at 180°. Operating conditions: column temperature, isothermal at 115°; detector temperature, 300°; injection temperature, 200°; gas flow-rate, carrier gas helium at 20 ml/min at 50 p.s.i., hydrogen pressure at 40 p.s.i., air pressure at 50 p.s.i., Beckman recorder, pen response at 0.5-sec full scale, a high accuracy of ±0.25%, and a sensitive 1-mV range. The sensitivity was 0.5×10^{-10} a.f.s. for most analyses.

Reagents

The following reagents were used: L-Epinephrine (Calbiochem, La Jolla, Calif., U.S.A.); norepinephrine base (—)-arterenol, bitartrate hydrate (Calbiochem, San Diego, Calif., U.S.A.); tetrahydrofuran (THF), silylation grade (Pierce, Rockford, Ill., U.S.A.); BSA (Supelco, Bellefonte, Pa., U.S.A.); trifluoroacetic anhydride (TFA) (PCR, Gainesville, Fla., U.S.A.); *n*-hexane, 99 mole% pure certified (Fisher Certified Reagent; Fisher Scientific, Fair Lawn, N.J., U.S.A.); and 4-methyl-2-pentanone (Matheson Coleman and Bell, Norwood (Cincinnati), Ohio, U.S.A.).

Procedure

Preparation of standards. Standard reference catecholamines were prepared by weighing exactly 10.0 mg of E and 10.0 mg of NE base (arterenol, bitartrate hydrate). The standards were transferred into separate 10-ml volumetric flasks and dissolved in 0.025 *N* hydrochloric acid. The standard reference were diluted in 0.025 *N* hydrochloric acid, 1.0 ng/μl. Aliquots of these solutions were used to obtain detection limits of the catecholamines under these conditions. The internal reference marker tripalmitin, grade approx. 99% (Sigma, St. Louis, Mo., U.S.A.), was prepared by weighing 20.0 mg of the tripalmitin. This reference marker was transferred to a 10-ml volumetric flask, dissolved in chloroform, and made up to volume with the same solvent. This internal reference solution was diluted to a concentration of 2.0 ng/μl.

Blood plasma and serum preparation for catecholamines. On different occasions 5 ml whole blood were withdrawn from a subject into a 10-ml syringe and immediately transferred to a 10-ml heparinized test tube containing 0.5 mg sodium metabisulfite per milliliter whole blood. The blood sample was mixed well and kept in an ice-bath at all times until we were ready to separate the plasma from the red blood cells by centrifugation. The blood sample was transferred into a thick-walled 15-ml plastic centrifuge tube. The plasma and red blood cells were separated by centrifuging at 25,000 *g* at 5° in a Sorvall RCZ-B centrifuge for 10 min.

The plasma was transferred by a Pasteur pipette to a 50-ml plastic centrifuge tube, and the exact volume of the sample was recorded. Cold 70% perchloric acid (PCA) was added to the test tube —0.044 ml per ml of plasma or serum. The test

tube was covered with a plastic cap and mixed vigorously on the vortex mixer for 1 min; after this, the plasma–PCA mixture stood at 5° for at least 25 min to complete protein precipitation. The mixture was then centrifuged at 25,000 *g* at 5° for 20 min; the volume of the supernatant was determined and transferred to a 5-ml volumetric flask. The protein-free filtrate was made up to total volume with 0.4 *N* PCA.

Aliquots of the supernatant were transferred to a vacutainer-evacuated glass test tube (127 × 16 mm), placed in a heating block set at 50°, and evaporated with a stream of nitrogen to complete dryness. The derivatives were prepared from the residue for GLC analysis.

Red-blood-cell preparation. The red blood cells (RBC) from the plasma separation were used for the RBC catecholamines: 0.5 ml of RBC were pipetted into a 50-ml plastic centrifuge tube, and 1.5 ml of deionized water and 0.250 ml of 70% PCA were added. The tube was mixed thoroughly and centrifuged at 25,000 *g* at 5° for 20 min. After centrifugation the volume of the supernatant was determined and transferred to a 2-ml volumetric flask. The protein-free filtrate was made up to volume with 0.4 *N* PCA.

Aliquots of the supernatant were transferred to a vacutainer-evacuated glass test tube (127 × 16 mm), placed in a heating block set at 50°, and dried as far as possible with a stream of nitrogen. The residue that remained was extracted with 0.5 ml of deionized water, 0.5 ml of *n*-hexane, and 0.5 ml of 4-methyl-2-pentanone. The top organic layer of the two-phase extract was removed and transferred to a separate test tube and dried under a stream of nitrogen. The derivatives were prepared from the residue for GLC analysis.

Urine preparation. In collecting the urine samples, the time of bladder evacuation was noted and the milliliters of urine collected per minute were calculated. After adding 0.5 mg of sodium metabisulfite per milliliter of urine and 0.15 ml of 70% perchloric acid, 5 ml of the urine sample were frozen for subsequent analysis. (The highly basic urine must achieve at least pH 4.0 before storage.) For assay, the urine samples must be thawed and mixed well. The urine–perchloric acid mixture was centrifuged at 25,000 *g* at 5° for 10 min to remove the solids. An aliquot of the clear supernatant was transferred to a vacutainer-evacuated (non-anticoagulant) glass test tube, placed in a heating block set at 50°, and dried with a stream of nitrogen. Derivatives were prepared from the residues for GLC analysis.

Catecholamine-derivative preparation. The residues containing catecholamines were dissolved in 0.1 ml of BSA, adding 0.1 ml of THF and 0.1 ml of TFA, and mixed thoroughly. The mixture was placed in a heating block for 10 min at 50°. The derivatives were washed with 1.0 ml of deionized distilled water, 1.0 ml of *n*-hexane, and 0.5 ml of 4-methyl-2-pentanone; this mixture was thoroughly mixed for 1 min on a vortex mixer, and the phases were allowed to separate. The top layer of the two-phase extract was removed and transferred to vacutainer-evacuated test tubes and dried as far as possible with a stream of nitrogen. The residues were dissolved in 0.1–0.2 ml of 4-methyl-2-pentanone for use in GLC analysis.

Calibration curves. The standards were prepared in 4-methyl-2-pentanone to contain 0.13×10^{-3}–1.7×10^{-3} ng of NE and 0.1×10^{-3}–2.4×10^{-3} ng of E; injection volume was 2.0 μl. Before injection, a flush volume of 1 μl of 4-methyl-2-pentanone was taken into the syringe before each sample. The standards were injected into the gas chromatograph. Calibration curves of each catecholamine were prepared

from the chromatographic data. The average peak heights of each reference standard GC peak were plotted against the catecholamine concentrations.

Peak height measurement. Peak height measurement is more rapid than peak area measurement; however, plots of peak height *vs.* sample size have a more limited linear range than corresponding plots for peak area. Peak heights and widths depend on sample size and sample feed volume, but peak area does not. Peak heights are used where the samples are less than 10 μg for packed columns and 0.1 μg for capillary columns.

The peak heights used were measured in millimeters, as the distance from baseline to peak maxima. The concentration of the catecholamine standard was known and the factor was calculated as:

$$\text{factor (ng/mm)} = \frac{\text{(ng injected)}}{\text{(mm)}}$$

The concentration of the standard and/or unknown catecholamine was calculated:

$$\text{ng catecholamine per } \mu\text{l} = \frac{\text{(factor)} \times \text{(mm)}}{(\mu\text{l injected})}$$

RESULTS AND DISCUSSION

In the method described, a dual flame ionization detector was used in the GLC analysis of the catecholamines extracted from the biological fluids. According to other workers flame ionization is not as sensitive as an electron capture detector, but it is less easily contaminated. The linearity of the dual flame ionization response to catecholamines extends over a much wider range of concentration than does that obtained with an electron capture detector.

In the gas chromatographic analysis of biogenic amines in biological material, these amines must be transformed into derivatives with greater volatility and lower polarity than those of the parent compound. In catecholamine analysis, the investigator normally deals with unstable substances in dilution of 10^{-6} or less.

Preliminary investigation of the catecholamines, analyzed by GLC on 7% DC-11 on 80–100 mesh Gas-Chrom P stationary phase, produced chromatograms with no tailing. In the GLC analysis described here, the catecholamines derivatives are prepared with THF–TFA–BSA (1:1:1) and chromatographed on a column of 7% DC-11 on 80–100 mesh Gas-Chrom P.

The column was conditioned at 180° for a minimum of 48 h with carrier-gas flow, and the temperature reduced to 115° for all analyses.

The efficiency of the 7% DC-11 column for the catecholamines was obtained from the number of theoretical plates in 4-methyl-2-pentanone. The column demonstrated an apparent efficiency of theoretical plates for E, NE, and tripalmitin of 296, 264 and 619, respectively.

Linear detector response was obtained by establishing an absolute calibration curve of each catecholamine (see Fig. 3, bottom chromatogram). Standard solutions of catecholamines were prepared over the concentration range of approx. 0.0001–0.0024 ng. In each case the maximum of 3.0-μl samples were injected into the gas chromatograph.

Table I shows the relative retention times for the GLC analysis of the standard catecholamines and those found in blood plasma, red blood cells, serum, and urine.

Table II shows the reproducibility of peak heights of E and NE derivatives on the day of the analysis and over a period of three days on single standard samples.

TABLE I

RETENTION TIMES OF COMPONENTS IN STANDARD MIXTURES, BLOOD PLASMA, RED BLOOD CELLS, SERUM, AND URINE

Column temperature, 115°; carrier gas flow-rate, 20 ml/min.

Compound	*Relative retention time (min)**				
	Standard	*Plasma*	*RBC*	*Serum*	*Urine*
Epinephrine**	0.507	0.508	0.518	0.500	0.530
Norepinephrine***	1.16	1.16	1.14	1.16	1.17
Tripalmitin§	1.00	1.00	1.00	1.00	1.00

* Retention time relative to tripalmitin.
** 3,4-Dihydroxy-N-methylphenylethanolamine.
*** 3,4-Dihydroxyphenylethanolamine.
§ Glyceryl tripalmitate (internal reference marker).

TABLE II

REPRODUCIBILITY OF VALUES ON PEAK HEIGHTS OF EPINEPHRINE AND NOREPINEPHRINE DERIVATIVES

Compound	*No. of determinations*	*Mean peak height (mm)*	*S.D.*	*CV (%)**
E (1 day)	3	20.3	0.58	2.86
NE (1 day)	3	17.3	0.64	3.70
E (3 days)	3	21.5	1.32	6.14
NE (3 days)	3	17.9	0.71	3.97

* Coefficient of variation = $\frac{\text{S.D.}}{\text{mean}} \times 100$.

TABLE III

RECOVERY OF EPINEPHRINE AND NOREPINEPHRINE ADDED TO PLASMA, SERUM, RED BLOOD CELLS, AND URINE

Sample	*Amount added (ng × 10⁻³)*		*Amount* found (ng × 10⁻³)*			
	E	*NE*	*E*	*% Recovered*	*NE*	*% Recovered*
Plasma**	1.25	1.13	1.20 ± 0.12	96.0	1.09 ± 0.09	96.5
Serum***	2.50	1.50	2.31 ± 0.01	92.4	1.63 ± 0.06	108.7
Red blood cells**	2.50	2.50	2.74 ± 0.10	109.6	2.19 ± 0.43	87.6
Urine***	3.75	3.75	3.65 ± 0.42	97.3	3.37 ± 0.43	92.3

* Means of two to nine determinations.
** Guinea pig.
*** Human.

TABLE IV

CATECHOLAMINES IN PLASMA, RED BLOOD CELLS, SERUM, AND URINE

Column temperature, 115°; carrier gas flow-rate, 20 ml/min.

Compound	*Plasma (ng/ml)**	*RBC (ng/ml)**	*Serum (ng/ml)**	*Urine (ng/min × 10^3)*
Epinephrine	0.386 ± 0.041	0.364 ± 0.16	0.326 ± 0.03	19.7 ± 0.09**
	—	—	—	32.2 ± 0.05***
Norepinephrine	0.331 ± 0.05	0.466 ± 0.35	0.118 ± 0.14	3.1 ± 0.03**
	—	—	—	5.4 ± 0.02***

* Means of two to seven determinations.
** 0.25 ml of urine.
*** 0.5 ml of urine.

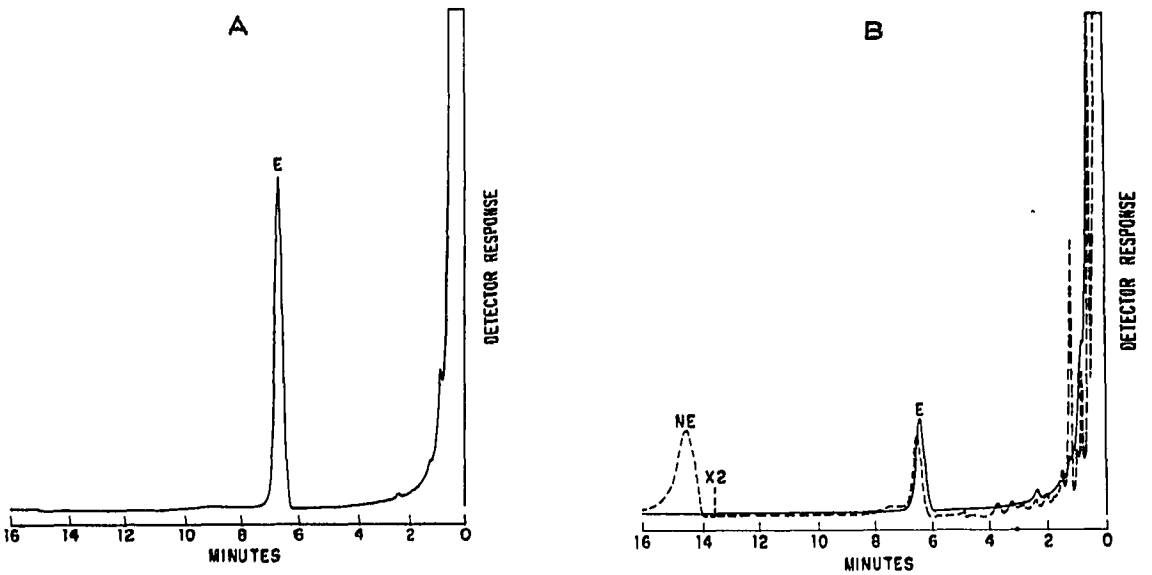

Fig. 1. A, Gas chromatogram of a standard of E in 4-methyl-2-pentanone. B, Gas chromatogram of a standard of E (solid line) and a mixture of E and NE in 4-methyl-2-pentanone (superimposed as broken lines). Sample size: 1.0 µl of E and 1.0 µl of E + NE. Operating conditions: column, 7% DC-11 on 80–100 mesh Gas-Chrom P; temperature, column isothermal at 115°, injection 200°, flame detector 300°; sample size, 1.8 µl.

All samples analyzed on the same day and over a period of three days by the GLC method, for both E and NE derivatives, gave a coefficient of variation of 6.14% or less.

Catecholamine recoveries are shown in Table III. Standards were added to plasma, red blood cells, serum, and urine. Extractions were carried out as stated in this text and analyzed by GLC.

Table IV shows the individual concentration of catecholamines found in plasma, red blood cells, serum, and urine. The values for urine are expressed in nanograms per minute.

Fig. 1A shows gas chromatogram peaks of standard E in 4-methyl-2-penta-

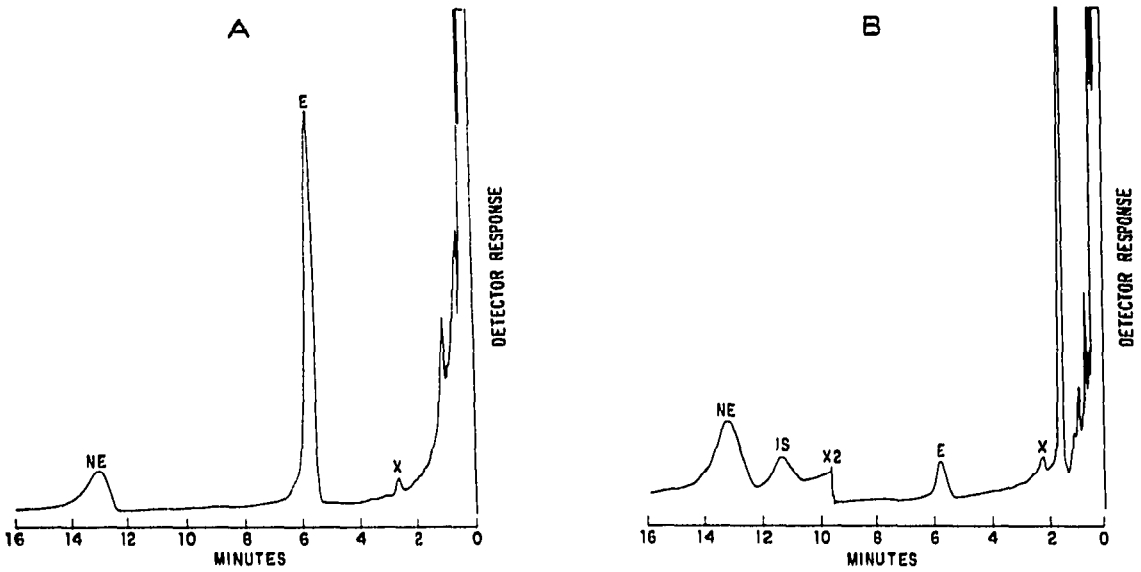

Fig. 2. A, Gas chromatogram of a fraction of plasma–4-methyl-2-pentanone extract of E, NE, and an unidentified peak (X). B, Gas chromatogram of a fraction of plasma E and NE and a reference marker, tripalmitin (IS), in 4-methyl-2-pentanone; X, X2, unidentified peaks. GLC conditions as described in the legend to Fig. 1.

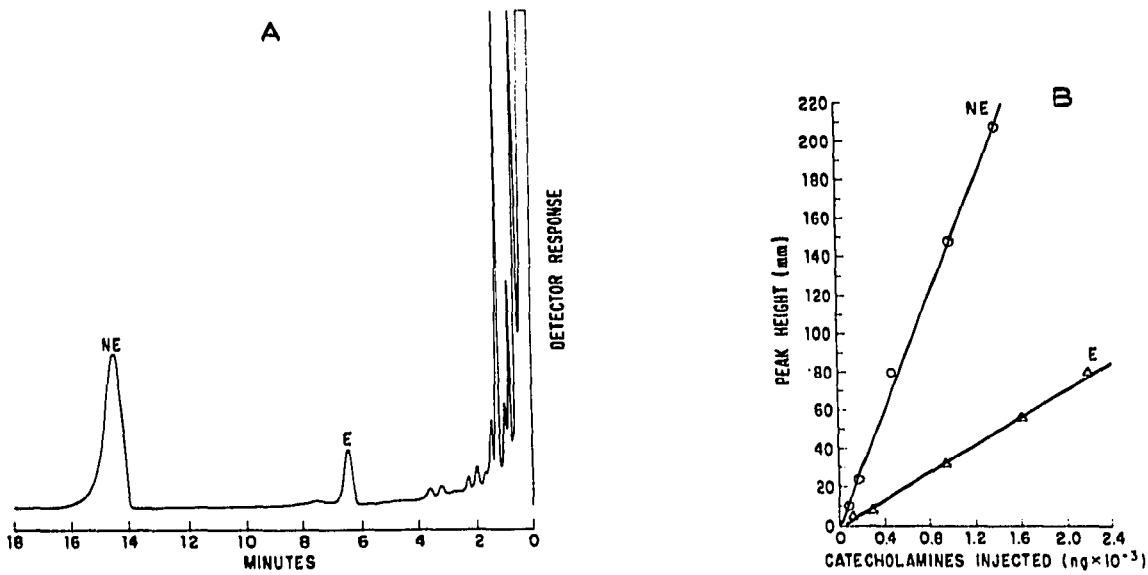

Fig. 3. A, Gas chromatogram of a fraction of E and NE added to the plasma and carried through extraction and separation procedures for recovery analysis. B, Calibration curves of injected E and NE. GLC conditions as described in the legend to Fig. 1.

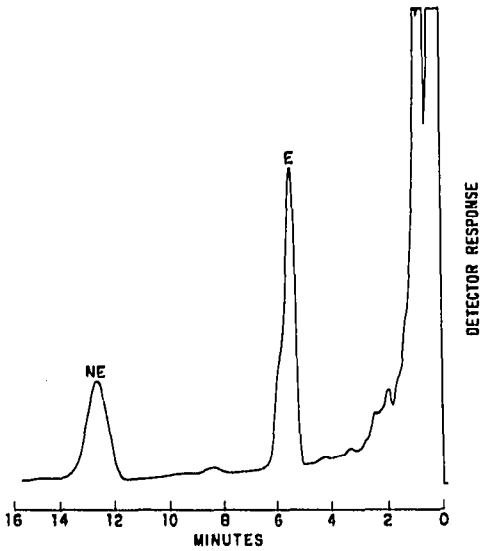

Fig. 4. Gas chromatogram of a fraction of red blood cell–4-methyl-2-pentanone extract of E and NE. GLC conditions as described in the legend to Fig. 1.

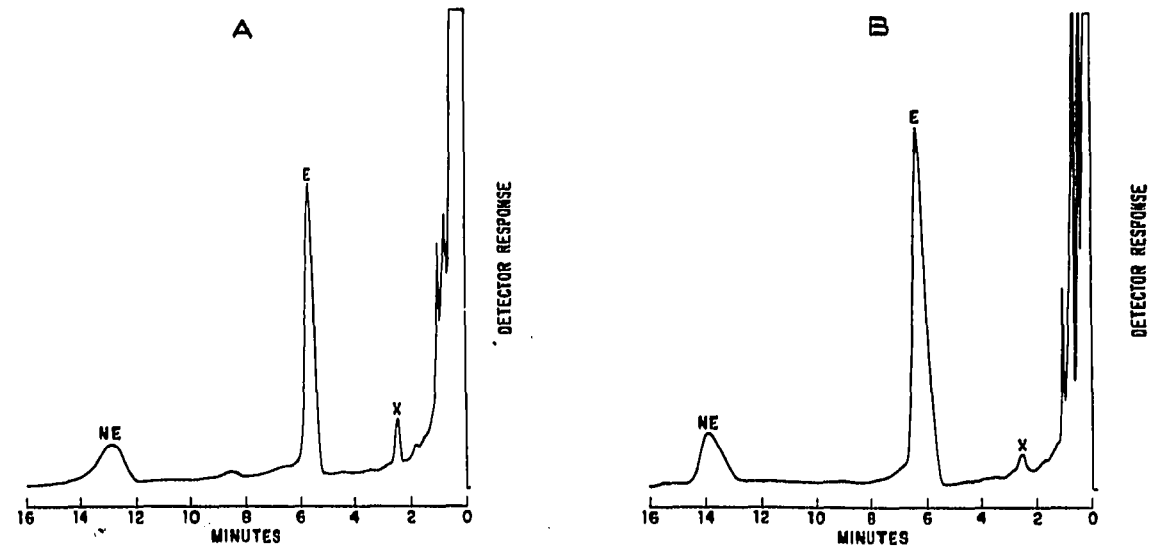

Fig. 5. A, Gas chromatogram of a fraction of human serum–4-methyl-2-pentanone extract of E, NE, and an unidentified peak (X). B, Gas chromatogram of a fraction of human urine–4-methyl-2-pentanone extract of E, NE, and an unidentified peak (X). GLC conditions as described in the legend to Fig. 1.

none. E has a retention time of approximately 6.1 min and a relative retention time to tripalmitin of 0.5 (Table I). In Fig. 1B, a standard mixture of E and NE in 4 methyl-2-pentanone is shown superimposed on the E curve; they separate completely with no tailing of the peaks. Fig. 2 shows chromatogram peaks of E and NE obtained from

plasma–4-methyl-2-pentanone extract. Fig. 2B shows the catecholamines (E, NE) with the internal reference marker (IS); X is unidentified. Fig. 3A shows the gas chromatogram obtained by adding E and NE to blood plasma and carrying out the extraction procedure for recovery analysis. Fig. 4 shows chromatogram peaks of E and NE obtained from red blood cells in 4-methyl-2-pentanone extract. Fig. 5A and B shows chromatogram peaks of E and NE obtained from human serum and urine.

Selection of the internal reference marker is important. It should be inert toward all sample components; it may or may not be chemically similar, although the former is preferred; and it should be completely resolved from all other peaks. Our results show that the relative retention time ratios were constant for the pure and unknown mixtures.

In plasma and red blood cells, E and NE concentrations are very low, and their estimation requires that methods be used at the limit of their sensitivity. The method presented in this report has been successful in studying plasma, red blood cells, serum, and urine containing E and NE, as our detection limit for the catecholamines with the hydrogen flame detector was approximately 0.1 pg.

The quantitative determination of E, dopamine and NE in human red blood cells will be discussed in a separate report. Mass spectra, NMR, and IR analyses are under way for identification of the characteristic fragmentation, spectral bands and characteristic ion spectra.

ACKNOWLEDGEMENT

The research reported in this paper was conducted by personnel of Biodynamics Branch, Environmental Sciences Division, USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, United States Air Force, Brooks AFB, Texas. Further reproduction is authorized to satisfy the needs of the U.S. Government.

REFERENCES

1 P. Capella and E. C. Horning, *Anal. Chem.*, 38 (1966) 316.
2 N. P. Sen and P. L. McGeer, *Biochem. Biophys. Res. Commun.*, 13 (1963) 390.
3 E. Brochmann-Hanssen and A. B. Svendsen, *J. Pharm. Sci.*, 51 (1962) 938.
4 S. Linstedt, *Helv. Chim. Acta*, 41 (1958) 1915.
5 A. C. Moffat and E. C. Horning, *Biochim. Biophys. Acta*, 222 (1970) 248.
6 M. G. Horning, A. M. Moss and E. C. Horning, *Biochim. Biophys. Acta*, 148 (1967) 597.
7 M. G. Horning, A. M. Moss and E. C. Horning, *Anal. Biochem.*, 22 (1968) 597.
8 S. Kawai and Z. Tamura, *Chem. Pharm. Bull.*, 15 (1967) 1493.
9 S. Kawai and Z. Tamura, *J. Chromatogr.*, 25 (1966) 472.
10 S. Kawai, T. Nagatsu, T. Imanari and Z. Tamura, *Chem. Pharm. Bull.*, 14 (1966) 618.
11 S. Kawai and Z. Tamura, *Chem. Pharm. Bull.*, 16 (1968) 699.
12 K. Imai, M. Sugiura and Z. Tamura, *Chem. Pharm. Bull.*, 19 (1971) 409.
13 Kim Ping Wong, C. R. J. Ruthven and M. Sandler, *Clin. Chim. Acta*, 47 (1973) 215.
14 T. L. Sourkes and B. D. Drujan, *Can. J. Biochem. Physiol.*, 35 (1957) 711.
15 K. Imai, Mei-Tai Wang, S. Yoshiue and Z. Tamura, *Clin. Chim. Acta*, 43 (1973) 145.