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PRE-COLUMN DERIVATIZATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BIOGENIC AMINES IN BLOOD OF NORMAL AND MALIGNANT HYPERTHERMIC PIGS*

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SUMMARY

A sensitive, selective, pre-column derivatization method was used with high-performance liquid chromatography to measure norepinephrine, dopamine and serotonin in plasma from normal and malignant hyperthermic (MH) pigs. Samples were carefully collected from control and stressed animals under halothane anesthesia. Using a simple extraction method involving pre-column derivatization with *o*-phthalaldehyde and ethyl acetate partitioning, the samples were chromatographed in less than 50 min.

Norepinephrine was found to be elevated in MH pigs as the syndrome progressed, reaching levels eight-fold greater than control pigs under anesthesia. These experiments provide some evidence for our hypothesis that a failure to metabolize excess norepinephrine may be one of the key metabolic defects in causing the pathophysiology of the malignant hyperthermia—stress syndrome.

The application of our chromatographic method in animal and human tests may provide a pattern of biogenic amine types and levels that could be diagnostic in identifying susceptible humans and carrier animals.

INTRODUCTION

Our laboratory has been involved for several years in developing a highly

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sensitive chromatographic method for the analysis of biogenic amines in biological materials and introduced first the concept of pre-column derivatization followed by high-performance liquid chromatographic (HPLC) separation. We established the pre-column derivatization conditions, recovery, and linearity of the method [1—5]. The major obstacles that we earlier encountered in analyzing biogenic amines were the lability of the native molecules, the difficulty with a selective and efficient extraction from aqueous biological samples, and the optimization of the separation conditions. After trying a number of different approaches, including solvent extraction of underivatized amines, ion-exchange, alumina adsorption, and gas—liquid chromatography (GLC), we found that derivatization of the amines with *o*-phthalaldehyde (OPA)—mercaptoethanol reagent in the aqueous sample stabilized the molecules for a selective and efficient solvent extraction which we then coupled with HPLC separation. With this approach we were able to extract and chromatograph a spectrum of biogenic amines at the same time, in contrast to those procedures which allow only 2 or 3 molecules to be assayed [6—11]. In addition, the derivatization yielded highly fluorescent adducts 20 times more intense than the native compounds [12]. This feature helped to overcome the problem of detecting the nanogram levels of biogenic amines present in plasma samples. With the enhanced sensitivity of this new method, samples of only 2 ml of plasma could be used routinely in the analysis. This small sample size is in contrast to other fluorescent methods which require a much larger amount of sample [6—8]. Over the years, the trihydroxyindole method has been used by many researchers and published widely [6, 8, 13]; however, this method has major limitations of sensitivity, selectivity, lack of precision and accuracy, and is very time consuming. Other important advantages of the new approach to the analysis of biogenic amines are rapid and simple sample cleanup with good recovery at low levels. Again, this is in contrast to the alumina adsorption or Biorex 70 cleanup procedures which often yield less than desirable recoveries at low levels and have poor precision and accuracy. When coupled with the high efficiency of the HPLC separation, the presented method gives excellent confidence in the identity of each of the individual molecules. Later, Mell et al. [14] used this concept of pre-column derivatization in the analysis of norepinephrine and dopamine in urine.

The idea that norepinephrine and other biogenic amines play a dominant role in the development of the fulminant hyperthermia—stress syndrome has been presented on a theoretical basis and partially substantiated with catecholamine depletor, false transmitter, and alpha blocker studies [15—19]. However, the crucial test of the norepinephrine hypothesis is to accurately quantitate key biogenic amines in blood plasma during the syndrome. This paper reports on the results we have obtained by using our HPLC separation method which is selective, specific and sensitive.

METHODS AND MATERIALS

Susceptible pigs were raised from our strain of susceptible stock maintained at the Sinclair Experimental Medicine Research Farm. Control animals were purchased from a local producer. The animals were premedicated and

prepared for these experiments by inserting a French 8 catheter in the external iliac vein.

Sample collection

Whole blood (20 ml) was rapidly collected in a sterile all-glass 20-ml syringe which was rinsed on the inside with sterile saline—heparin solution. The collected blood was then emptied immediately into an ice water chilled 25-ml screw cap glass centrifuge tube (Scientific Products, St. Louis, MO, U.S.A.) which contained 1 ml of freshly made EDTA—metabisulfite solution (20 mg/ml EDTA, 10 mg/ml sodium metabisulfite). The tube was capped with a PTFE-lined screw cap and inverted gently several times to mix the blood and reagent, then taken to a cold room (4°C) and immediately spun for 10 min at 4000 *g*. The plasma was removed from the packed cells and the volume measured at the same time with a 10-ml all-glass syringe with a Kel-F Ever hub and 3-in PTFE-tube (Scientific Products). Working in the cold room, the volume of plasma was recorded, made 0.4 mol/l with concentrated perchloric acid, mixed, and then the tube was allowed to stand in the cold for 15 min to complete perchlorate precipitation of protein. After precipitation, the sample was centrifuged at 20,000 *g* for 20 min at 4°C, removed and the supernatant immediately stored at —70°C in the dark.

Apparatus

A Model 6000A solvent delivery system and U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) were used with the HPLC system. The fluorescence detector was a SpectrofluoroMonitor Model FS970 with a 5-μl flow-cell and selectable monochromatic excitation wavelength (Schoeffel Instruments, Westwood, NJ, U.S.A.). The recorder was a Fisher Recordall Model 5000 (Houston Instruments, Austin, TX, U.S.A.). The HPLC columns used for reversed-phase HPLC were packed with 10-μm particles and were μBondapak phenyl, 300 mm × 4 mm (Waters Assoc.) columns. A pre-column, Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was used to protect the analytical column.

The temperature of the HPLC column was controlled by a constant-temperature circulating water bath Model FJ (Haake, Saddle Brook, NJ, U.S.A.), connected to an aluminum column jacket.

Peak areas, retention times, relative weight response (RWR) values, and concentrations based on an internal standard were calculated by a Hewlett-Packard 3352B Laboratory Data System (Hewlett-Packard, Avondale, PA, U.S.A.). The sytem consists of a Hewlett-Packard 2100 computer with 24K memory, 18652A analog-to-digital converters (A/D), ASR33 teletype, and a 2748B high-speed reader.

Chemicals

All reagents used were of highest purity available (A.C.S. certified grade). Buffers and aqueous reagent solutions were prepared with all-glass-distilled, reagent grade, Nanopure water.

Monobasic sodium phosphate (A.C.S. certified grade; Fisher Scientific,

Fairlawn, NJ, U.S.A.), methanol, glass-distilled (Burdick and Jackson Labs., Muskegon, MI, U.S.A.), were used to prepare buffers.

Boric acid, 2-mercaptoethanol, and potassium hydroxide (A.C.S. certified grade; Fisher Scientific) and *o*-phthalaldehyde (Sigma, St. Louis, MO, U.S.A.) were used to prepare the derivatization reagent.

Sodium metabisulfite, disodium ethylenediaminetetraacetate (EDTA), dibasic sodium phosphate, perchloric acid (A.C.S. certified and reagent grade; Fisher Scientific), and ethyl acetate (spectrophotometric grade; Aldrich, Milwaukee, WI, U.S.A.) were used in the preparation of the samples for HPLC analysis.

Histamine (HI), norepinephrine (NE) and octopamine (OCT) were purchased from Sigma. Dopamine (DA) and tyramine (TYM) were purchased from Aldrich, normetanephrine (NMN) and serotonin (5-HT) from Calbiochem (San Diego, CA, U.S.A.). The standards used were the hydrochlorides of the amines, dissolved in methanol, and stored in glass containers at −20°C. Under those conditions the standard solutions were stable for over six months.

Sample extraction for HPLC

The samples were thawed in flowing water at 20—30°C, mixed thoroughly, and a 2-ml aliquot was pipetted into a 12-ml conical culture tube. The deproteinated sample was then adjusted to a pH of 7.0 ± 0.2, with 0.5 mol/l potassium hydroxide and immediately derivatized with 400 µl (350 µg) of *o*-phthalaldehyde buffered at pH 10.40 ± 0.02. Sodium chloride (2 g) was added to break any emulsion formed during the double extraction with 2 ml of ethyl acetate. The sample was shaken for 1 min during each extraction and spun at 3400 *g* to separate the phases. After extraction the ethyl acetate was partitioned twice with 2 ml of 35 mmol/l dibasic sodium phosphate buffer, pH 10.0 ± 0.1, shaken for 1 min, and spun at 3400 *g*. The final ethyl acetate volume was reduced to 100 µl under a sweep of ultra-pure, oxygen-free, dry nitrogen gas and stored in the cold room (4°C) until analysis. This procedure resulted in a recovery of 70% for all biogenic amines studied with a relative standard deviation of 6% at physiological concentration. Under these conditions the derivatives were stable for more than 24 h with no detectable changes in peak height or area [4, 5]. This enables one to prepare samples in advance, which could significantly increase sample output.

The ethyl acetate extract containing the derivatized biogenic amines was brought to room temperature and 10—50 µl were injected and chromatographed with a NaH_2PO_4 buffer (0.025 mol/l, pH 5.10), containing 25% acetonitrile per liter for the first elution step and 450 ml methanol (45%) for the second elution step. The chromatography was performed with a flow-rate of 1.5 ml/min. The derivatives were quantitated by measuring their fluorescence intensity at 340 nm excitation and at 480 nm emission.

Biogenic amine confirmation

The biogenic amines were identified on the basis of retention time by comparison with reference compounds and also by co-chromatography of reference compounds in different solvent systems such as various concentrations of methanol, tetrahydrofuran, and acetonitrile [5].

In addition, the identity of NE and DA was confirmed by correlating the values obtained from the trihydroxyindole determination to those by *o*-phthalaldehyde HPLC analysis of the same rat brain samples [4].

RESULTS

Fig. 1 describes the separation of the *o*-phthalaldehyde derivatives at the nanogram level. We were able to accomplish a detection limit of less than 100 pg due to the pre-column derivatization of the biogenic amines. As shown in Figs. 2 and 3, we applied the separation to a study of normal pigs and pigs afflicted with the genetic disorder of malignant hyperthermia (MH). Note the large increase of NE in the plasma from the malignant hyperthermic pig versus the control pig.

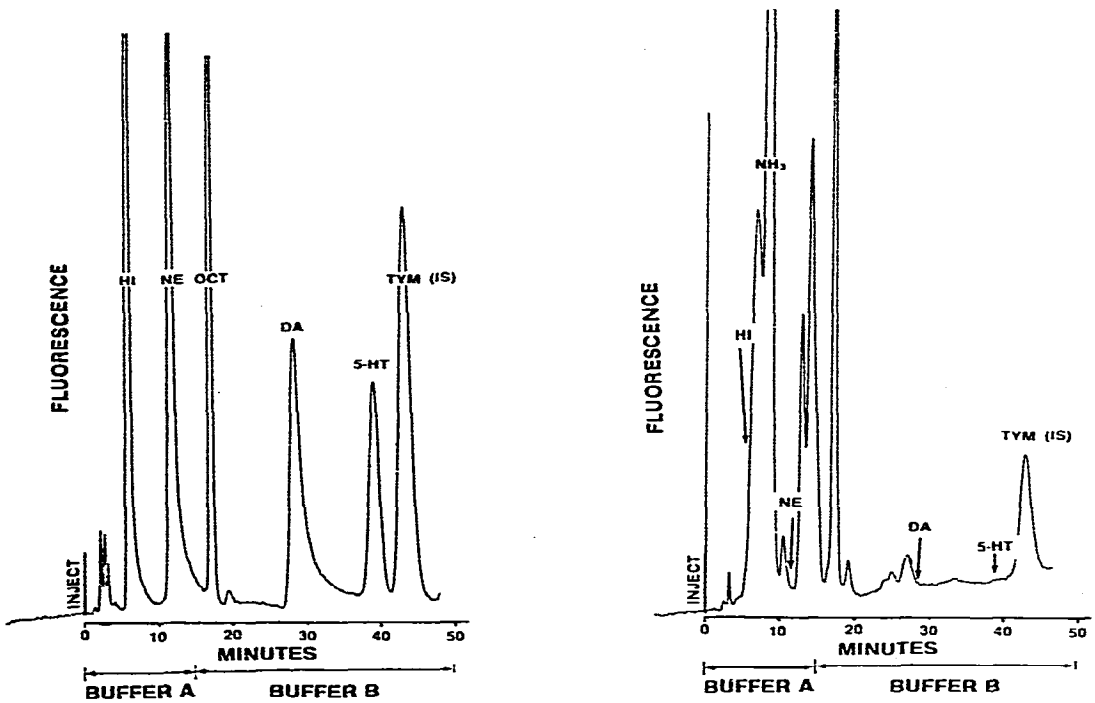

Fig. 1. Reversed-phase HPLC separation of seven biogenic amine standards. Sample, 10 μl standards, 10 ng each; column, μBondapak phenyl (300 × 4 mm); buffer, 0.025 mol/l NaH_2PO_4 (pH 5.10) with 250 ml of acetonitrile per l for first elution step (A) and 450 ml of methanol per l for second elution step (B); flow-rate, 1.5 ml/min; detector, Schoeffel FS970, 0.10 μA full-scale, excitation 340 nm, emission 480 nm; temperature, 26°C. The internal standard (IS) is octopamine. Peaks: HI, histamine; NE, norepinephrine; OCT, octopamine; NMN, normetanephrine; DA, dopamine; 5-HT, serotonin; TYM, tyramine.

Fig. 2. Reversed-phase HPLC separation of biogenic amines in plasma from a control pig. Sample, 10 μl, equivalent to 0.20 ml plasma; column, μBondapak phenyl (300 × 4 mm); buffer: 0.025 mol/l NaH_2PO_4 (pH 5.10), with 250 ml acetonitrile added per l for first elution step (A) and 450 ml of methanol added per l for second elution step (B); flow-rate, 1.5 ml/min; detector, Schoeffel FS970, 0.10 μA full-scale, excitation 340 nm, emission 480 nm; temperature, 26°C. Internal standard (IS) is tyramine because no indigenous tyramine was detected in the pig plasma. Abbreviations as in Fig. 1. Concentration of NE and DA are below 0.5 ng/ml.

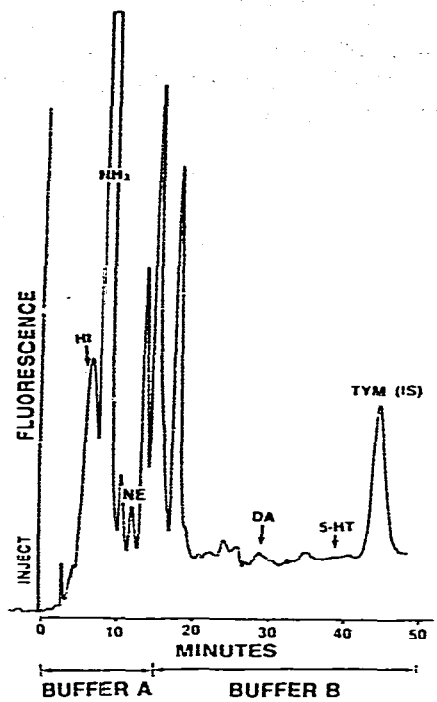

Fig. 3. Reversed-phase HPLC separation of biogenic amines in plasma from a malignant hyperthermic pig. Sample, 10 µl, equivalent to 0.20 ml plasma. All other conditions as in Fig. 2. Abbreviations as in Fig. 1. The concentration of NE in this separation is 0.9 ng/0.2 ml or 4.5 ng/ml.

TABLE I

BIOGENIC AMINE LEVELS IN CONTROL SWINE UNDER ANESTHESIA

Sample*	Biogenic amines, ng/ml plasma		
	NE	DA	5-HT
CW81-8-1	<0.5	4.9	6.0
2	<0.5	6.7	<0.5
3	<0.5	9.2	<0.5
4	0.6	8.0	2.8
CWO-0-1	<0.5	< 0.5	<0.5
2	<0.5	1.5	<0.5
3	<0.5	6.1	2.2
4	<0.5	4.1	3.2
D9-3-1	<0.5	< 0.5	0.8
2	<0.5	1.8	<0.5
3	<0.5	< 0.5	<0.5
P-27-4-1	<0.5	< 0.5	<0.5
2	<0.5	< 0.5	<0.5
3	<0.5	< 0.5	<0.5
P-29-7-1	<0.5	< 0.5	1.9
2	<0.5	10.4	<0.5
3	<0.5	4.0	<0.5
4	<0.5	7.0	<0.5

*Sample 1 = Control (no halothane); 2 = 15 min, 3 = 30 min, 4 = 45 min after halothane.

Table I contains the data from five control pigs under halothane anesthesia, and Table II the results for six MH pigs. Note that all MH pigs have significantly higher circulating levels of the mammalian vasoconstrictor NE at the height of the MH syndrome which was after 45 min under halothane anesthesia, than the control pigs after 1 h of anesthesia. It is also interesting to note that the NE levels increased at least eight-fold during the most acute stages of intense peripheral vasoconstriction and body temperature rise (Figs. 4 and 5). Serotonin levels were more variable and higher in some of the MH pigs than in the control animals. Dopamine levels in MH swine did not change during the course of the MH syndrome, however, this may be due to the fact that much of the plasma DA is conjugated and would therefore not be analyzed by our procedure. This is also demonstrated in the erratic values for concentrations reported in normal pigs. A detailed study of the hemodynamic parameters and body temperature changes for this particular group of pigs was published separately [20]. It appears from our data that the longer the MH positive pig is under halothane anesthesia the more intense the NE increases (Table II). The NE response reaches a peak after 45 min of anesthesia where the levels are 5- to 20-fold higher than controls (Tables I and II).

TABLE II

BIOGENIC AMINE LEVELS IN MALIGNANT HYPERTHERMIC SWINE UNDER ANESTHESIA

Sample*	Biogenic amines, ng/ml plasma		
	NE	DA	5-HT
P-10-6-1	< 0.5	4.6	20.2
2	< 0.5	8.6	< 0.5
3	4.0	5.3	< 0.5
4	14.9	5.2	2.1
P-16-3-1	< 0.5	4.3	< 0.5
2	0.8	8.5	< 0.5
3	6.4	3.8	< 0.5
4	7.5	4.4	< 0.5
P-16-9-1	< 0.5	5.4	1.1
2	< 0.5	9.8	< 0.5
3	4.5	9.7	< 0.5
4	2.6	13.6	< 0.5
P-21-3-1	1.2	5.4	0.6
2	0.8	5.0	0.8
3	1.9	< 0.5	12.1
4	1.4	< 0.5	2.8
P-22-5-1	< 0.5	< 0.5	< 0.5
2	2.2	2.4	2.9
3	1.4	4.8	8.6
4	2.1	< 0.5	2.2
P-23-2-1	< 0.5	0.8	< 0.5
2	< 0.5	< 0.5	< 0.5
3	37.0	< 0.5	0.8
4	5.7	< 0.5	1.2

*Sample 1 = Control (no halothane); 2 = 15 min after halothane given; 3 = at start of MH symptoms, 30 min after halothane; 4 = at height of MH syndrome, 45 min after halothane.

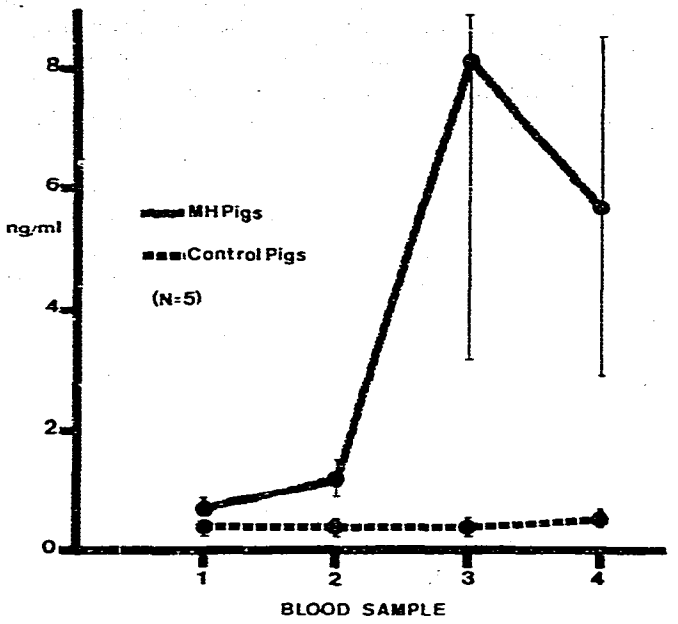

Fig. 4. Norepinephrine in plasma of malignant hyperthermic and control pigs before, sample 1, and under anesthesia, samples 2, 3, and 4. See also Tables I and II for other conditions.

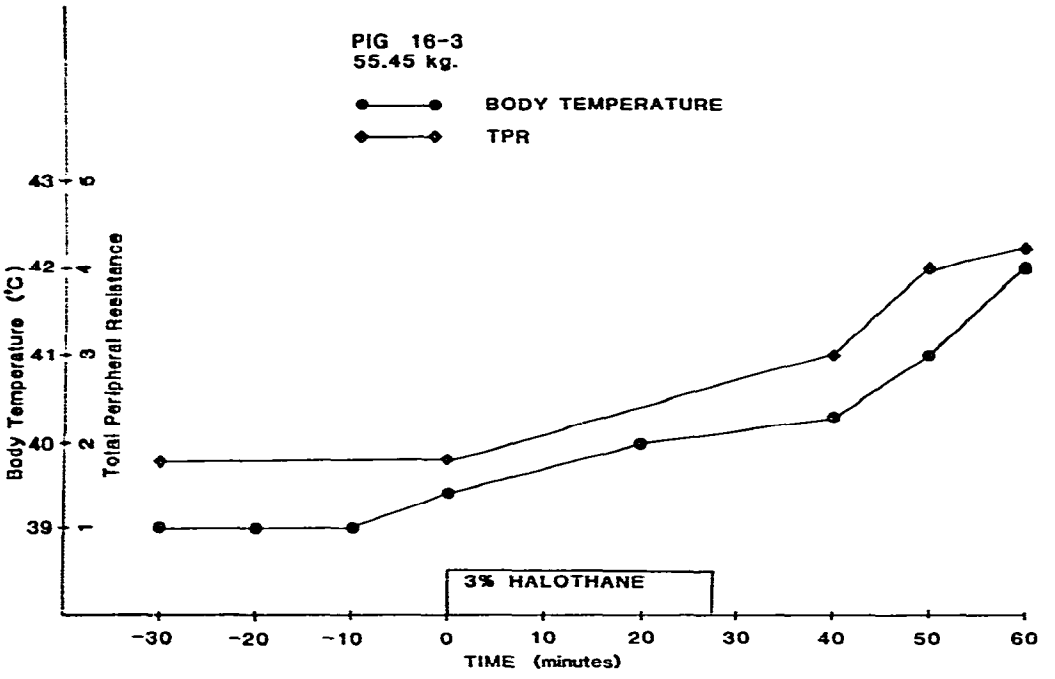

Fig. 5. Total peripheral resistance (TPR) and rectal temperature as a function of the development of the malignant hyperthermia syndrome in pig P-16-3.

DISCUSSION

The assumption that an excess activity of norepinephrine could be the primary cause of the malignant hyperthermia—stress syndrome was based on the observed pathophysiology of the syndrome [15—17, 20] and studies with α-methyl DOPA, reserpine, and phentolamine [18, 19]. The results presented in this paper provide support for our hypothesis and indicate that a failure to catabolize excess norepinephrine may be one of the key metabolic defects which causes the pathophysiology of the malignant hyperthermia—stress syndrome.

Studies of the catecholamines in blood and urine by other investigators have led to the publication of data that neither support nor refute the hypothesis that NE plays a dominant role in causing the malignant hyperthermia syndrome [21—25]. This could be due to the fact that they used the trihydroxyindole procedure which is not as selective, specific or sensitive as the presented HPLC method [1—5].

In addition to the new methodology that we have used in our research, we also inbred the MH pigs to enhance the genetic defect to its most potent form. In these animals a marked increase of NE was observed in the blood during the development of the syndrome. Unrelated swine of a different breed were used by us as controls. Other investigators have preferred to use halothane-negative litter mates as controls. However, we found that a halothane-negative animal at eight weeks of age can become halothane-positive at a later date; thus, the use of halothane-negative litter mates as control is unreliable.

More information could be gained by expanding our norepinephrine hypothesis by including other biogenic amines. The application of our chromatographic method for the analysis of pig and human blood may provide a pattern of biogenic amines and levels that could be diagnostic in identifying susceptible humans and carrier animals.

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REFERENCES

1 T.P. Davis. C.W. Gehrke, Jr., C.W. Gehrke, K.C. Kuo, C.H. Williams, H.D. Johnson and K.O. Gerhardt, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 36 (1977) 407.
2 T.P. Davis, C.W. Gehrke, Jr., T.D. Cunningham, C.W. Gehrke, K.C. Kuo and K.O. Gerhardt, American Chemical Society Midwest Regional Meeting, Rolla, MO, November 3—4, 1977.
3 T.P. Davis, C.W. Gehrke, T.D. Cunningham, K.O. Gerhardt, K.C. Kuo and C.W. Gehrke, Jr., Fed. Proc., Fed. Amer. Soc. Exp. Biol., 37 (1978) 1351.
4 T.P. Davis, C.W. Gehrke, C.W. Gehrke, Jr., T.D. Cunningham, K.C. Kuo, K.O. Gerhardt, H.D. Johnson and C.H. Williams, Clin. Chem., 24 (1978) 1317.
5 T.P. Davis, C.W. Gehrke, C.W. Gehrke, Jr., T.D. Cunningham, K.C. Kuo, K.O. Gerhardt, H.D. Johnson and C.H. Williams, J. Chromatogr., 162 (1979) 293.
6 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360.

7 S. Natelson, J.K. Lugovoy and J.B. Pincus, Arch. Biochem., 23 (1949) 157.
8 H. Weil-Malherbe and A.D. Bone, Biochem. J., 51 (1952) 311.
9 K. Engelman, B. Portnoy and W. Lovenberg, Amer. J. Med. Sci., 255 (1968) 268.
10 H. Ben-Jonathan and J.C. Porter, Endocrinology, 98 (1976) 6.
11 R.P. Maickel and F.P. Miller, Anal. Chem., 38 (1966) 12.
12 S.S. Simons and D.F. Johnson, J. Amer. Chem. Soc., 98 (1976) 7098.
13 T. Nagatsu, Biochemistry of Catecholamines, University of Tokyo Press, Tokyo, Japan, 1973.
14 L.D. Mell, A.R. Dasler and A.B. Gustafson, J. Liquid Chromatogr., 1 (1978) 261.
15 C.H. Williams, in E.O. Henschel (Editor), Animal Models of Malignant Hyperthermia. Malignant Hyperthermia: Current Concepts, Appleton Century Crofts, New York, 1977, pp. 117—140.
16 C.H. Williams, in E.O. Henschel (Editor), The Development of an Animal Model for the Fulminant Hyperthermia—Stress Syndrome. Malignant Hyperthermia: Current Concepts, Appleton Century Crofts, New York, 1977, pp. 117—140.
17 C.H. Williams, Perspect. Biol. Med., 20 (1976) 120.
18 C.W. Short, R.R. Paddleford, C.J. McGrath and C.H. Williams, Anesth. Analg. Cleveland, 55 (1976) 643.
19 C.H. Williams and D.H. Stubbs, in P. Lomax and E. Schonbaum (Editors), Preliminary Studies on the Therapeutic Efficacy of Phentolamine in the Fulminant Hyperthermia—Stress Syndrome. Drugs, Biogenic Amines and Body Temperature, Karger, Basel, 1976, pp. 243—244.
20 C.H. Williams, M.D. Shanklin, H.B. Hedrick, M.E. Muhrer, D.H. Stubbs, G.F. Krause, C.G. Payne, J.D. Benedict, D.P. Hutcheson and J.F. Lasley, in A.G. Aldrete and B.A. Britt (Editors), The Fulminant Hyperthermia—Stress Syndrome: Genetic Aspects, Hemodynamic and Metabolic Measurements in Susceptible and Normal Pigs. 2nd Int. Symp. Malignant Hyperthermia, Denver, April 1—3, 1977, Grune and Stratton, New York, 1978, pp. 113—140.
21 G.A. Gronert, J.H. Milde and R.A. Theye, Anesthesiology, 47 (1977) 411.
22 G.M. Hall, J.N. Lucke and D.A. Lister, Anaesthesia, 30 (1975) 308.
23 T.G. Althen, K. Ono and D.G. Topel, J. Anim. Sci., 41 (1975) 284.
24 M.P. Judge, E.J. Briskey, R.G. Cassens, J.C. Forrest and R.K. Meyer, Amer. J. Physiol., 214 (1968) 146.
25 D.M. Altrogge, D.G. Topel, M.A. Cooper, J.W. Hallberg and D.D. Draper, J. Anim. Sci., 51 (1980) 74.