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APPLICATION OF A NOVEL CATION-EXCHANGE REAGENT, IGEPON T-77 (N-METHYL OLEOYL TAURATE), TO MICROBORE SEPARATIONS OF ALUMINA EXTRACTS OF CATECHOLAMINES FROM CEREBROSPINAL FLUID, PLASMA, URINE AND BRAIN TISSUE WITH AMPEROMETRIC DETECTION

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SUMMARY

The use of a novel amide surfactant, N-methyl oleoyl taurate (Igepon T-77), has been examined for the separation of amines on reversed-phase chromatographic material. This reagent was found to partition onto the C_{18} material in a partially irreversible and concentration independent manner. When the stationary phase is saturated with this surfactant, the loaded column performs as a strong cation exchanger. Novel separations are possbile as a result of secondary hydrogen-bonding effects which modify classical retention order for primary, secondary and tertiary amines. Sensitive and selective applications of these separations are demonstrated for catecholamine determinations in blood plasma, cerebrospinal fluid, urine and brain tissue. Additional sensitivity is obtained for epinephrine by taking advantage of the pH-dependent intramolecular cyclization and on-column concentration of large injection volumes.

INTRODUCTION

Reversed-phase separations for biogenic amines have been widely published. In general, these separations are adaptations of ion-pairing techniques [1–8], using alkyl sulfates or sulfonates to modify reversibly the reversed-phase surface, enhancing retention of the cationic species. Selectivity is obtained by varying the ionic strength, organic modifier (methanol, acetonitrile, etc.) or ion-pair reagent concentration. The reversed-phase properties of the stationary phase are generally retained such that separation of neutral or relatively non-polar compounds may also be accomplished. The advantages of this approach are obvious as this allows simultaneous separation and detection of amines and neutral or acidic metabolites in a single sample injection [1]. In some analytical situations, how-

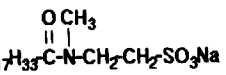

Fig. 1. Igepon T-77, N-methyl oleoyl taurate, sodium salt.

ever, the mixing of separation mechanisms (ion-pair/exchange, ion suppression and reversed-phase) can prove to be a disadvantage in terms of chromatographic specificity. These situations, such as analysis of plasma catecholamines, are sometimes better approached by using ion exchange, which relies primarily on a single mechanism for separation [9].

We have recently examined a number of surfactants with novel properties for the separation of biogenic amines [10,11]. For the past year one of these compounds, Igepon T-77 (N-methyl oleoyl taurate, Fig. 1), has been extensively evaluated for selectivity in separation of biogenic amines. The novel properties imparted to C_{18} reversed-phase material by this reagent have been combined with microbore high-performance liquid chromatography (HPLC) and amperometric detection to achieve extremely sensitive and selective methods for determination of catecholamines in a variety of matrices. Sample preparation is a straightforward batch aluminum oxide procedure [19] and large volumes (50 μl) of the extract may be applied to the column taking advantage of the "non-eluting matrix" approach. Detection limits of less than 1 pg/ml for epinephrine are obtained for analysis of 1-ml samples of cerebrospinal fluid (CSF) or plasma. Absolute detection limits of 0.2 pg (1 fmol) per injection are obtained for epinephrine.

EXPERIMENTAL

Chemicals and reagents

Igepon T-77 was obtained from GAF Corporation (Wayne, NJ, U.S.A.). Biogenic amines, epinephrine, norepinephrine, dopamine, 3,4-dihydroxybenzyl amine, serotonin and N-methylserotonin were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were reagent grade. Water was distilled and deionized.

Apparatus

Reversed-phase C_{18} microbore columns were prepared as previously described [13]. Columns were 10 cm$\times$1.1 mm I.D. packed with 3-μm C_{18} ODS Shandon Hypersil (Alltech Assoc., Deerfield, IL, U.S.A.). Pulseless solvent delivery for the microbore system was accomplished using an Applied Chromatography Systems Model 400-02 pump (Peris Industries, State College, PA, U.S.A.). Sample injection was accomplished using a Gilson Model 231 autosampler (Thomson Instruments, Springfield, VA, U.S.A.) fitted with either a 20- or 50-μl fixed volume injection loop.

Detection was accomplished amperometrically at a glassy carbon electrode (TL-8A, Bioanalytical Systems, West Lafayette, IN, U.S.A.) using an EC/230 amperometric liquid chromatographic detector (IBM Instruments, Danbury, CT, U.S.A.). A silver/silver chloride reference electrode was used. Column dead volume was determined, when necessary, by observing the first detector deflection

following injection of sample as this reflected the elution of unretained ions, causing a detectable change in background current due to the change in solution resistance.

Samples

Plasma, urine and CSF were obtained in the course of ongoing clinical studies. Plasma was generally collected using EDTA as anticoagulant although at times heparin was also used. Urine was collected into bottles containing sodium metabisulfite as reducing agent and CSF was collected without preservative and frozen immediately. All samples were stored at −80 °C for varying periods prior to analysis. Rat brain tissue samples were obtained as part of ongoing pharmacological studies.

RESULTS AND DISCUSSION

Novel chromatographic properties of Igepon T-77

This surfactant, like a similar amide carboxylic acid surfactant, lauroyl sarcosine, semi-irreversibly "loads" the C_{18} reversed-phase material [11]. The concentration of Igepon T-77 in the mobile phase determines the length of time required to obtain saturation of the reversed-phase material. As a result, "loading" the column can be accomplished quickly by applying a high-concentration mobile phase containing 10 g/l Igepon T-77 for 30 min to achieve saturation, then applying the analytical mobile phase. In this manner, equilibrium can be obtained in approximately 1 h. Overnight (8–12 h) equilibration is required to obtain saturation using the analytical mobile phase which usually contains 100–250 mg/l Igepon T-77. No discernable difference in retention of biogenic amines is observed with mobile phase concentrations of Igepon T-77 between 0.1 and 1 g/l. Once the column has been loaded, use of a mobile phase containing no surfacant is possible for several hours without observable effect on separation, however, 100–250 mg/l is generally included to assure equilibrium saturation of the reversed-phase material.

As was previously observed with both lauroyl sarcosine [11] and Tween nonionic surfactant [10], saturation of the stationary phase with Igepon T-77 appears to eliminate reversed-phase sites. As a result, polar neutrals and carboxylic acids, which can be separated simultaneously when alkyl sulfate–sulfonate surfactants are used in conjunction with ion suppression [1], are unretained.

The separation of amines obtained using Igepon T-77 is governed by mobile phase ionic strength and methanol content as shown in Fig. 2. pH has a negligible effect on capacity factor as is the case for alkyl sulfate/sulfonate surfactants in the useful pH range for this silica-based stationary phase.

The most interesting and most useful properties imparted to C_{18} reversed-phase material with respect to separation of biogenic amines are shown in Fig. 3. The overall order of elution of catecholamines is governed by polarity, with less polar primary amines eluting later. However, for a homologous series, the order of elution is governed by the bond order of the amine. This is shown for both catecholamines and indoleamines. Capacity factors for the primary amines are greater than for the respective secondary amines which are in turn greater than for the

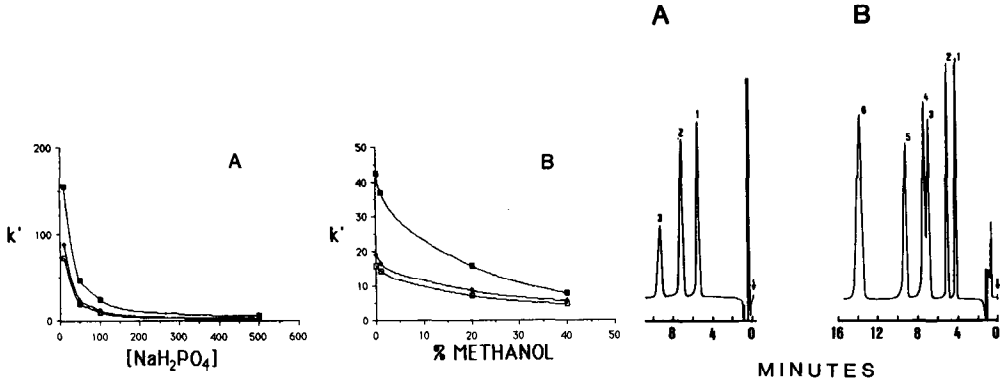

Fig. 2. (A) Effect of solvent ionic strength on capacity factors of catecholamines. Column parameters were as in B. Solvent consisted of varying concentrations of sodium hydrogen phosphate and 600 mg/l Igepon T-77. Concentration units are m*M*. (B) Effect of mobile phase methanol content on capacity factors of catecholamines. Column was 7.5 cm × 4.6 mm I.D. packed with 3-μm ODS Hypersil. The mobile phase was 50 m*M* sodium dihydrogenphosphate, 600 mg/l Igepon T-77 and was maintained at a flow-rate of 1.0 ml/min. Approximately 1 h was allowed for equilibrium between each concentration of methanol, or until repeated injections were of constant retention time. □ = Epinephrine; ♦ = norepinephrine; ■ = dopamine.

Fig. 3. Effects of Igepon T-77 on retention order of biogenic amines. (A) Separation of indoleamines. Peaks: 1 = bufotenine (N,N-dimethyl-5-hydroxytryptamine); 2 = N-methyl-5-hydroxytryptamine; 3 = 5-hydroxytryptamine. Column was 10 cm × 1.1 mm I.D. packed with 3-μm ODS Hypersil. Solvent was 0.3 *M* sodium acetate-methanol (85:15), containing 250 mg/l Igepon T-77, pH 7.0. Flow-rate was 100 μl/min. (B) Separation of catecholamines. Peaks: 1 = epinephrine (N-methyl-3,4-dihydroxyphenylethanolamine); 2 = norepinephrine (3,4-dihydroxyphenylethanolamine); 3 = epinine (N-methyl-3,4-dihydroxyphenylethylamine, N-methyldopamine); 4 = 3,4-dihydroxybenzylamine; 5 = dopamine (3,4-dihydroxyphenylethylamine); 6 = α-methyldopamine (α-methyl-3,4-dihydroxyphenylethylamine). Column was 10 cm × 1.1 mm I.D. packed with 3-μm ODS Hypersil. Mobile phase was 0.15 *M* sodium acetate-methanol (85:15), 150 mg/l Igepon T-77, pH 7.0. Flow-rate was 100 μl/min.

tertiary amines. The effect is the same as was observed when alkyl sulfates were used simultaneously with Tween non-ionic surfactants [10]. This secondary effect on retention order was attributed to hydrogen bonding in the former case. A similar explanation may be appropriate in the present case since an amide bond is present two methylene groups removed from the anionic site. It is not likely that the observed effect is due to differences in the pK_a for the catecholamines or indoleamines as these are only modestly different, in the 8.80–8.95 range [13]. The inversion of retention order, compared to classical ion-pair separations of these compounds, is observed across the pH range tested, 2–7.5. This effect on retention order is a particularly useful feature in the selective enhancement of the relative amperometric signal for epinephrine, because of the low relative concentrations of epinephrine compared to norepinephrine found in most tissues or body fluids. Elution of epinephrine first adds a modest [$1/(1+k')$] enhancement in the relative peak height and decreases the resolution required to measure accurately the peak.

The second asset of this surfactant in the separation of these amines is the closeness of the relative capacity factors for the catecholamines while maintaining "large" capacity factors for the earliest eluting catecholamines. Conventional alkyl sulfates/sulfonates give a much wider range of capacity factors such that optimum resolution of norepinephrine from solvent front in complex samples such as plasma extracts leads to excessive analysis times for dopamine [14,15]. Igepon T-77 allows timely separation of all three catecholamines from such samples while giving excellent resolution from the solvent front.

Because this reagent saturates the reversed-phase sites on the stationary phase, polar neutral and acidic metabolites are not retained under the conditions used for the analytical applications which follow. The Igepon T-77 loaded column performs essentially as a strong cation exchanger under these conditions. These properties have been taken advantage of in order to apply relatively large sample volumes, as great as the void volume, to the column.

Electrochemical signal enhancement

In addition to chromatographic control of the peak concentration [16], the amperometric detector signal is dependent upon the mass transport rate of solution over the electrode, electrode area, the diffusion coefficient of the analyte and the number of electrons involved in the oxidation or reduction of the analyte [1,16,18]. Mass transport rate and electrode area can be modified mechanically to optimize the signal. The diffusion coefficients for the three catecholamines of interest are nearly equal and cannot be used to obtain any marked signal enhancement. One would generally assume that the number of electrons involved in the oxidation of these compounds is also fixed. Oxidation of the catecholamines is a two electron–two proton process to form the respective *ortho*-quinones [19]. At very low pH, the side-chain amines are protonated, however, at higher pH (>3) the fraction of unprotonated amine becomes significant. With increasing pH the fraction of unprotonated amine increases and with this the oxidized species is able to undergo an intramolecular 1,4-addition to form the respective reduced indole [13] (see Fig. 4). The rate of this reaction increases with increasing pH and is significantly faster for epinephrine than norepinephrine or dopamine. As a result, operating at a pH of around 7 leads to a significant enhancement of the epinephrine peak height by virtue of increasing the apparent number of electrons involved in the oxidation. This is demonstrated in Fig. 5. As can be seen, no signal enhancement is observed for dopamine relative to the internal standard while the signal for epinephrine is markedly enhanced. A modest increase in relative signal is observed for norepinephrine. There results are in complete agreement with the kinetic data [13].

These factors, relative elution order and increased apparent number of electrons from oxidation give roughly a three-fold relative signal enhancement for epinephrine versus norepinephrine when the present solvent system is compared to determination of these compounds by conventional liquid chromatography with electrochemical detection (LC–ED) using alkyl sulfate/sulfonate ion-pair reagents in a mobile phase with pH 3–5.

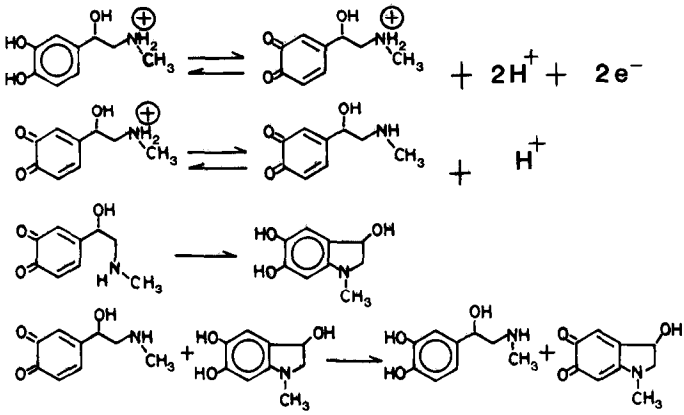

Fig. 4. Intramolecular cyclization of epinephrine *ortho*-quinone to regenerate oxidizeable epinephrine. (From ref. 13).

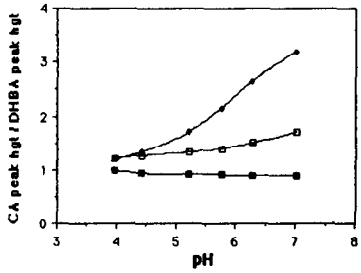

Fig. 5. Effect of pH on peak relative peak height of catecholamines (CAs). Measured peak heights of 25 pmol of each compound were compared to the peak height of 25 pmol of 3,4-dihydroxybenzylamine (DHBA), a catecholamine unable to undergo intramolecular cyclization. Solvent was 0.1 *M* sodium acetate–methanol (85:15), containing 250 mg/l Igepon T-77. pH was adjusted with acetic acid. Catecholamines: □ = norepinephrine; ♦ = epinephrine; ■ = dopamine.

Application to plasma and CSF catecholamine determinations

Plasma and CSF are two matrices in which analysis of catecholamines is quite difficult. We have previously applied microbore LC–ED to measurements of catecholamines in these fluids using a modified diphenylborate extraction [17] to obtain selectivity and preconcentration of the sample into a small volume suitable for application to the microbore column [20]. While this procedure has proved satisfactory and sensitive, the sample isolation and preconcentration requires multiple transfers and extractions. In addition, we and others [21] who have used this procedure have encountered great variability in batch to batch reproducibility of the cation-exchange material and diphenylborate reagent used in the extraction. The aluminum oxide batch procedure originally described by Keller et al. [22] adapted from earlier procedures [23] is widely used and has been shown to give reproducible results when combined with cation-exchange separation of catecholamines [9].

An aliquot of blood plasma or CSF (usually 1.0 ml) is transferred into a 1.5-ml polypropylene tube. To this is added a known quantity (25 pmol) of 3,4-

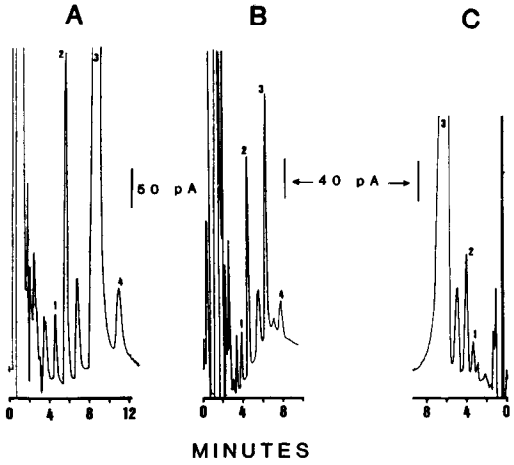

Fig. 6. Representative chromatograms of alumina extracts. (A) Human plasma catecholamines. A 40-μl volume of extract from 1.0 ml of plasma extracted as described in the text. Solvent was 0.1 *M* sodium acetate–methanol (85:15), containing 250 mg/l Igepon T-77, pH 7.0. Column parameters were the same as in Fig. 3. Applied potential was +0.55 V vs. Ag/AgCl reference. Flow-rate was 120 μl/min. (B) Human CSF catecholamines. A 40-μl volume of a 75-μl alumina extract from 1.0 ml of CSF applied to the column. Solvent conditions were 0.2 *M* sodium acetate–methanol (85:15), containing 250 mg/l Igepon T-77, pH 7.0. Flow-rate was 120 μl/min. Column parameters and electrode potential were the same as in (A). (C) Rat plasma catecholamines. A 40-μl volume of extract from 200 μl of rat plasma. Solvent, column and electrode parameters were as in (A). Peaks: 1 = epinephrine; 2 = norepinephrine; 3 = epinine; 4 = 3,4-dihydroxybenzylamine.

dihydroxybenzylamine as internal standard. To this is added 20 mg of acid washed aluminum oxide and 0.5 ml of 1.0 *M* Tris–buffer, pH 8.6. The mixture is shaken vigorously for 10–15 min and the supernatant discarded. The alumina is washed once with 1.5 ml of distilled deionized water and the wash discarded. The alumina is aspirated to dryness and 100 μl of 1% acetic acid are added to desorb the catecholamines. While other acids are frequently used for this step, we chose to use acetic acid because of the low degree of ionization and thus the low ionic strength. The clear supernatant obtained after centrifugation for 2–3 min at 12 800 *g* is removed and placed in a clean 1.5-ml polypropylene tube. Samples are then placed in the autosampler rack for application to the chromatographic system. Injection volumes are 25–50 μl. Fig. 6A–C shows representative chromatograms of extracts from human plasma, 200 μl of rat plasma and human CSF. These chromatograms were obtained over the course of the past year and represent results obtained with different columns and modestly different solvent conditions and flow-rates. The solvent system used in these cases was 0.1–0.2 *M* sodium acetate–methanol (85:15), containing 200–250 mg/l Igepon T-77, with the solvent pH adjusted to 6.8–7.0 with acetic acid. Most striking are the clarity and magnitude of the signals observed. In nearly all samples, all three catecholamines are readily detected. Coupling the traditional aluminum oxide extraction with the automatic injection system allows 80–100 samples to be processed daily.

The use of dilute acetic acid for desorption from alumina and application to

the HPLC column allowed injection of volumes normally applied to conventional columns with little or no observable band broadening due to the non-eluting matrix effect. Fig. 2 demonstrates the effect of ionic strength on elution of catecholamines. No special precautions in sample preparation were required in order to compensate for the size of the HPLC column. We did observe that the use of heparinized plasma samples at lower mobile phase pH (pH 4.5) caused rapid clogging of the columns with peak splitting. At present, we attribute this effect to the presence of heparin which appeared to precipitate under these conditions. Repeated analysis of 0.5-ml aliquots of a pooled CSF sample yielded an intra-assay precision (relative S.D., $n=5$) of 11.4, 4.4 and 10.2% for epinephrine (0.051 pmol/ml), norepinephrine (1.15 pmol/ml) and dopamine (0.089 pmol/ml), respectively. Inter-assay precision (relative S.D., $n=5$) was 15.5, 6.3 and 12.8%, respectively.

Application to determine of urinary catecholamines

Analysis of urinary catecholamines presents a somewhat different analytical problem compared to plasma or CSF catecholamines. Free catecholamine concentrations in urine are in the 10^{-6}–10^{-8} *M* range, well within the routine analytical range for conventional LC–ED. This differs from plasma or CSF catecholamines which are in the 10^{-9}–10^{-11} *M* range. Urine, however, presents a matrix in which the concentrations of numerous other electroactive phenolics are also quite high. Consequently most reversed-phase methods for determination of urinary catecholamines are preceded by multiple cleanup and isolation steps [21]. The strong cation-exchange properties imparted to the reversed-phase material by Igepon T-77 allow greater selectivity due to the coverage of reversed-phase sites. This allows a single batch alumina extraction for isolation of catecholamines prior to application to the HPLC column.

An aliquot of urine, 0.5–1.0 ml, is placed in a 1.5-ml polypropylene tube. To this are added 250 pmol of internal standard and 20 mg of acid washed aluminum oxide. The tube is then filled (0.5–1.0 ml) with 1.0 *M* Tris buffer, pH 8.6. Extraction is then accomplished following the same steps as used for plasma or CSF catecholamines. An injection volume of 10 μl is adequate for detection of urinary catecholamines. Fig. 7 shows a representative chromatogram of an alumina extract of urinary catecholamines. Repeated analysis of 0.5 ml of a pooled urine sample gave intra-assay precision (relative S.D., $n=5$) of 1.5–2.6% for all three catecholamines and less than 5% inter-assay variability ($n=5$).

Tissue catecholamine determination

Tissue catecholamines offer the least difficult analytical problems of the matrices discussed. There is a wide range of concentrations of the various catecholamines present in different brain regions yet the concentrations of dopamine and norepinephrine are generally well within the analytical range for conventional LC–ED techniques. Our special interest is in analysis of epinephrine in various brain regions which presents a more difficult analytical problem. Epinephrine concentrations in rat brain are quite low, even in areas of highest concentration [20]. In hypothalamus, epinephrine represents only about 2% of the total cate-

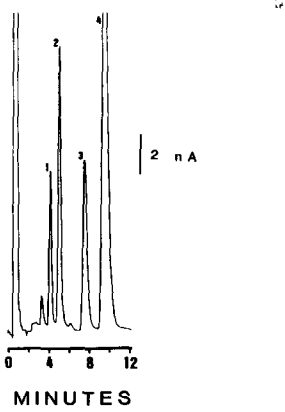

Fig. 7. Representative chromatogram of 25 μl of an alumina extract from 1.0 ml of human urine. Peaks as in Fig. 6. Solvent, column and electrode parameters as in Fig. 6A.

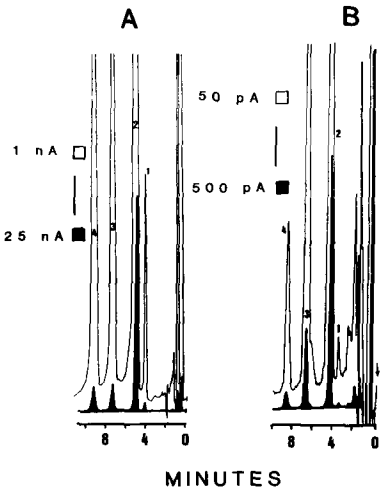

Fig. 8. Representative chromatograms of alumina extracts of (A) rat hypothalamus, and (B) rat cerebellum. A 25-μl volume was applied to the column. Peaks as in Fig. 6. Conditions as in Fig. 6B.

cholamines, 40–60 ng/g tissue vs. 2000 ng/g tissue for norephinephrine. A whole hypothalamus weighs about 25 mg. In other areas, concentrations of epinephrine are even lower and are in the 1 ng/g range in cerebral hemispheres or cerebellum. Discrete mapping of epinephrine in various nuclei requires both extremely low detection limits and complete resolution from norepinephrine which is present in a 50- to 100-fold excess in most areas. The order of elution obtained when using Igepon T-77 is quite fortuitous in solution of the latter requirement. The additional sensitivity obtained by use of microbore HPLC and injection of large volumes makes this chromatographic approach the most sensitive and selective technique presently available for these analyses.

Tissue preparation and isolation of catecholamines is accomplished in much the same manner as for plasma, CSF and urine. Frozen tissue is weighed into a 1.5-ml polypropylene tube. A 300-μl volume of 0.1 *M* perchloric acid and an

appropriate quantity of the internal standard, 3,4-dihydroxybenzylamine (10–200 pmol) are added to the tube and the tissue is disrupted by sonication. To this are added 20 mg of acid washed aluminum oxide and the remaining volume is filled with 1.0 *M* Tris buffer, pH 8.6. The remainder of the extraction procedure is the same as for plasma, CSF and urine. The injection volume is variable dependent upon the tissue catecholamine content. Fig. 8 shows representative chromatograms from rat hypothalamus, a brain area "rich" in epinephrine and cerebellum, a brain area with very little epinephrine.

The batch aluminum extraction used in these applications gave recoveries of 50–70% for each of the catecholamines [22]. Within a given sample, recoveries were within 3% for all the catecholamines.

CONCLUSIONS

The data presented in this manuscript describe a novel surfactant, Igepon T-77, which can be used to impart strong cation-exchange properties to reversed-phase material in situ. C_{18} material is loaded in a partially irreversible manner. Reversed-phase properties are minimal and polar neutral and acidic compounds are not retained. Because of the polar taurate functional group of Igepon T-77, separations of amines are governed by both polarity and hydrogen bonding. The elution order for a homologous series of amines is governed by hydrogen bonding with the capacity factors increasing in the order primary > secondary > tertiary. The utility of this reagent is demonstrated for the separation and determination of catecholamines in a variety of matrices after a simple batch alumina extraction. The novel properties imparted to the reversed-phase material allows for the use of the non-eluting matrix approach for injection of large volumes onto a microbore column and the use of simple alumina extraction for sample cleanup and isolation. Additional signal enhancement is observed by using a mobile phase of relatively high pH to take advantage of the intramolecular cyclization of the oxidized epinephrine and to some extent norepinephrine.

The use of this novel surfactant coupled with the use of microbore HPLC with amperometric detection offers an exquisitely sensitive, selective and simple approach to analysis of catecholamines in complex media.

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