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REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION OF LIPOPHILIC DOPAMINE ANALOGUES AND DETERMINATION OF BRAIN AND SERUM CONCENTRATIONS AFTER SAMPLE CLEAN-UP ON SMALL SEPHADEX G-10 COLUMNS

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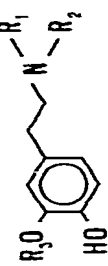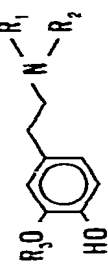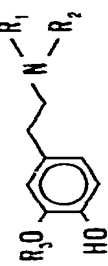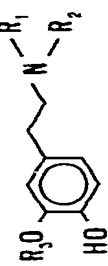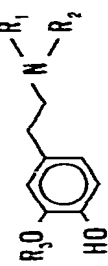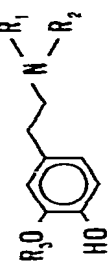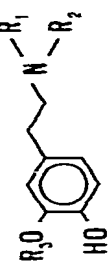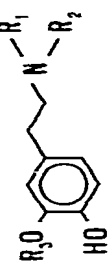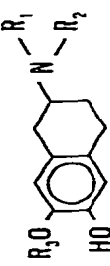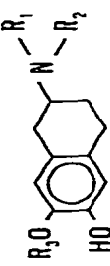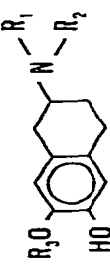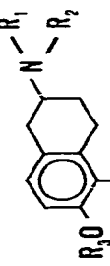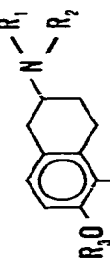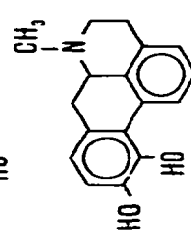
SUMMARY

The liquid chromatographic determination of N-alkylated analogues of dopamine is described. The retention and separation of these compounds, ranging from dopamine to N,N-dibutyl-dopamine, was studied on four bonded-phase columns, of which Nucleosil 5 C_{18} was chosen for routine use. The compounds were detected by a rotating disc amperometric detector. Samples of rat brain and serum were taken through a clean-up step on small Sephadex G-10 columns from which the dopamine analogues eluted in the same fraction as dopamine. The overall recovery was 70—90% from brain tissue and 60—70% from serum or plasma. The limit of detection for the catechol-containing compounds in tissue was 40—100 pg, for O-methylated ones 100--200 pg. The method is applied to the determination of dopamine analogues in rat brain after peripheral administration.

INTRODUCTION

High-performance liquid chromatography (HPLC) in combination with amperometric detection is now widely used for the determination of catecholamines and their metabolites in various tissues and body fluids [1—4]. Much less attention has been paid to the application of this technique to the analysis of compounds that are structurally related to catecholamines and that have been designed to exert a similar pharmacological action. Recent interest in dopaminergic drugs has led to the preparation of a series of dopamine (DA) receptor agonists which contain many of the structural features of DA itself [5—8]. Although the pharmacological properties of these compounds have

TABLE I

CHEMICAL FORMULAE OF THE COMPOUNDS USED

Compound	Abbreviation	Formula	R_1	R_2	R_3
Dopamine	DA		H	H	H
Epinine	Epi		CH_3	H	H
N,N-Dimethyl-dopamine	DiMe-DA		CH_3	CH_3	H
N,N-Diethyl-dopamine	DiEt-DA		C_2H_5	C_2H_5	H
N,N-Dipropyl-dopamine	DiPr-DA		C_3H_7	C_3H_7	H
N,N-Dibutyl-dopamine	DiBu-DA		C_4H_9	C_4H_9	H
3-Methoxy-tyramine	3-MT		H	H	CH_3
3-O-Methyl-N,N-dipropyl-dopamine	3-O-Me-DiPr-DA		C_3H_7	C_3H_7	CH_3
2-Amino-6,7-dihydroxy-tetralin	6,7-ADTN		H	H	H
2-N,N-Dimethylamino-6,7-dihydroxy-tetralin	DiMe-6,7-ADTN		CH_3	CH_3	H
2-N,N-Dipropylamino-6,7-dihydroxy-tetralin	DiPr-6,7-ADTN		C_3H_7	C_3H_7	H
2-Amino-6-hydroxy-7-methoxy-tetralin	7-O-Me-6,7-ADTN		H	H	CH_3
2-N,N-Dipropylamino-5,6-dihydroxy-tetralin	DiPr-5,6-ADTN		C_3H_7	C_3H_7	H
Apomorphine	APO		—	—	--

been studied extensively, in most cases the important information about distribution (for example, in the brain), metabolism and excretion is still lacking. This knowledge would be of great help in designing new centrally acting DA agonists that may be of value in the study and treatment of diseases such as Parkinsonism and schizophrenia [9].

The prototype of these compounds is apomorphine (APO), which is more lipophilic than DA itself and is able to penetrate the brain and activate central DA receptors [10,11]. APO has been assayed by various fluorimetric [12,13], gas chromatographic [14] and radioenzymatic [15] methods. Recently, HPLC methods have been described using spectrophotometric [16,17] or amperometric [18] detection. This last method can be applied for the determination of many DA analogues that possess a catechol group, and thus are easily oxidized. However, the potential of this method is only realized when used in combination with a reliable and fast sample clean-up procedure.

Isolation on small Sephadex G-10 columns was shown to be an excellent method for the isolation of catecholamines and their metabolites from brain tissue [4,19], cerebrospinal fluid [20] and urine [21]. In our laboratory we have been using this method for over two years in determining brain and serum concentrations of various DA agonists [18,22] and their metabolites [23] in experimental animals. The described methods were applied, except for APO, to rather hydrophilic compounds, that could be expected to show a chromatographic and electrochemical behaviour similar to DA.

In this article we report on the chromatographic behaviour, detection characteristics and sample clean-up of more lipophilic DA analogues of the DA and 2-aminotetralin series (Table I). We also describe the application of these methods for the determination of the DA analogues in rat brain and serum after peripheral administration.

METHODS

Materials

2-Aminotetralin and DA derivatives were synthesized according to known methods [24,25]. Dopamine · HCl (Serva, Heidelberg, G.F.R.) and apomorphine · ½H$_2$O · HCl (Brocades, Maarssen, The Netherlands) were commercially obtained. All liquids and reagents used were analytical grade and obtained from E. Merck (Darmstadt, G.F.R.). Water was deionized and glass-distilled.

Apparatus

Use was made of a Spectra-Physics 740 pump with a 740C pump control and 714 pressure monitor, and occasionally a Waters 6000A solvent delivery unit. Samples were injected by a Rheodyne 70-10 injection valve normally equipped with a sample loop of 200 μl.

Analytical columns (15 cm × 0.46 cm I.D.; Chrompack, Middelburg, The Netherlands) were packed with Nucleosil 5 C$_{18}$ (5 μm; Macherey-Nagel & Co., Düren, G.F.R.), LiChrosorb RP-2 (5 μm) and RP-8 (5 μm) (Merck) and Spherisorb S 5 Phenyl (5 μm; Phase Separations, Queensferry, Great Britain). Columns were packed using a Phase Sep packing bomb, a Waters 6000A pump

and suspensions of the materials in carbon tetrachloride—methanol (20:80). After packing the columns were purged with methanol and then water.

The detector was a rotating disc electrode [4,26], packed with carbon paste [made by carefully mixing 3.25 g of Spektralkohle RW-A (Ringsdorff) and 1.75 g of high vacuum silicon grease (DOW Corning)]. The surface of the electrode was normally polished on smooth paper for about 1 min. This was repeated when a high offset current or a decreased sensitivity appeared. The electrode was usually rotated with a speed of about 30 rps. The detector cell was further equipped with a platinum auxiliary electrode and a saturated calomel reference electrode. The detector was controlled by a potentiostat LC-2A (Bioanalytical Systems). The applied oxidation potential was 500 mV, except where otherwise indicated. Recordings of the chromatograms were made on a Kipp BD 41 recorder with a 0.5—5 V full-scale deflection. Sephadex G-10 columns (7 cm × 0.5 cm I.D.) were prepared in small pasteur pipettes as previously described [27].

Chromatography

Mobile phases consisted of McIllvaine buffer (0.1 M citric acid and 0.2 M Na$_2$HPO$_4$), with methanol or acetonitrile as organic modifiers. The buffer was filtered by suction through a 0.45-μm polycarbonate membrane filter (Schleicher and Schüll), after which methanol was added in a similar manner. This procedure provided sufficient degassing. EDTA (final concentration 10^{-4} M) was routinely added as its disodium salt. The flow-rate was usually 1.0 ml/min.

Chromatographic retention of the compounds was studied by varying the methanol and acetonitrile concentrations and the pH of the eluent. Retention is expressed as the capacity ratio $k' = (t_R - t_0)/t_0$, where t_R is the time from injection to the peak maximum and t_0 the time from injection to the first deflection in the detector response.

Separation factors for two compounds were calculated by dividing the capacity ratio of the last eluting compound by that of the first eluting compound. The number of theoretical plates was calculated as $n = 5.54(t_R/w_{0.5})^2$, where $w_{0.5}$ is the peak width at half-height.

Detection

The characteristics of the detector cell were studied by varying the distance between the surface of the rotating disc and the cell floor and by varying the rotation speed of the detector. Response was measured as peak height in nA. Noise in the detector response was measured as the spread in the baseline in nA. The susceptibility to oxidation of some compounds was studied by measuring the response in nA with varying oxidation cell potentials in mV, resulting in voltammograms.

Extraction procedures

Serum and brain tissues were taken from rats of a Wistar-derived strain (CDL, Groningen, The Netherlands). The animals were stunned and decapitated, and the brain tissues were rapidly removed, frozen on dry ice and kept at −80°C.

Blood, obtained by direct decapitation, was allowed to clot. Serum was prepared by centrifuging for 15 min at 1000 g and 4°C and stored at −80°C. Plasma was collected in heparinized tubes, centrifuged and stored as serum.

Tissue homogenates were prepared with a Potter-Elvejem homogenizer with a Teflon pestle in 5-ml glass tubes filled with 0.5 ml of 0.1 M perchloric acid (PCA). Homogenates were poured into polypropylene tubes. Pestle and glass tube were rinsed with another 0.5 ml of 0.1 M PCA, which was added to the homogenate. The homogenates were centrifuged at 3000 g and 4°C for 15 min. The supernatants were completely decanted into glass tubes, in which it was found they could be stored overnight at 4°C. Serum or plasma was deproteinized by mixing with 0.5 ml of 0.1 M PCA. After centrifugation the supernatants were completely decanted. The supernatants were applied to small Sephadex G-10 columns, which were prewashed with 2 × 1.5 ml of 0.02 M ammonia and 2 × 1.5 ml of 0.01 M formic acid (see Fig. 1) and were allowed to
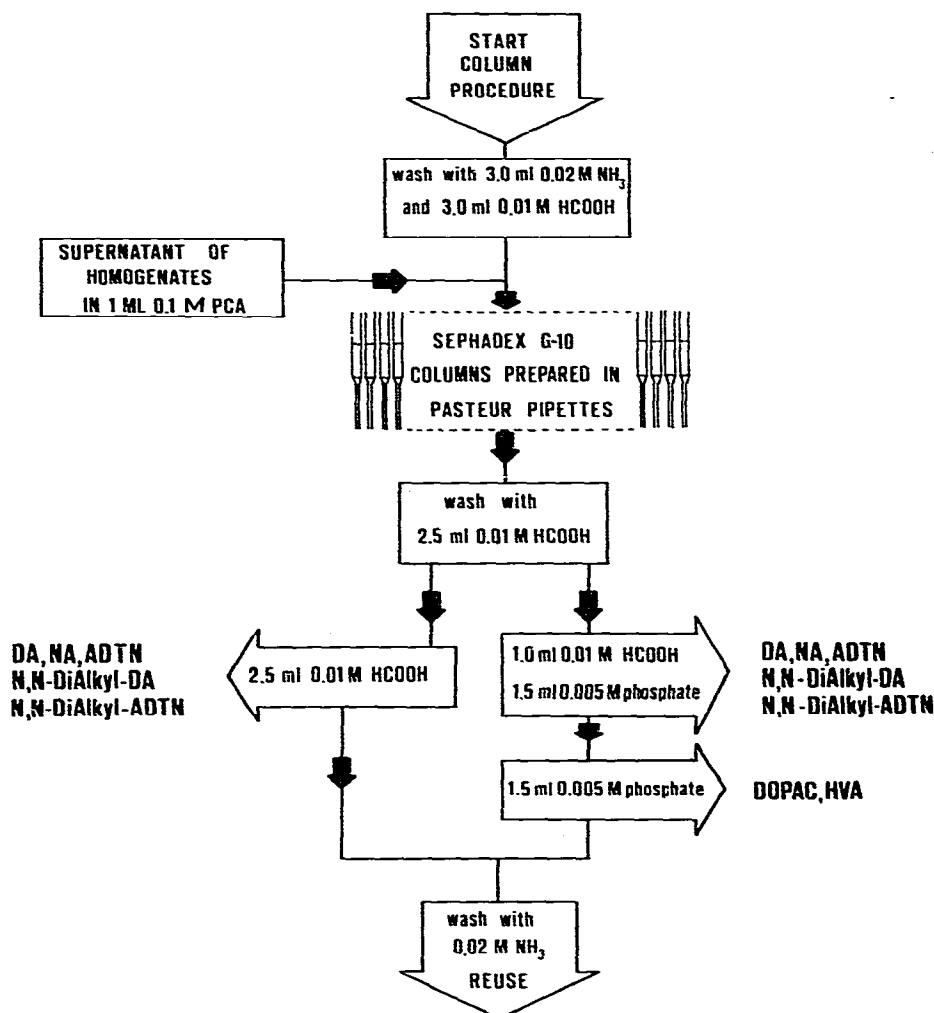
Fig. 1. Flow chart for the isolation procedure.

run dry. After application of the samples the columns were washed with 2.5 ml of 0.01 M formic acid and the amines were eluted with 2.0 or 2.5 ml of 0.01 M formic acid. When this elution is performed with 1.0 ml of formic acid and 1.5 ml of 0.005 M Na_2HPO_4, a second fraction may be collected which contains the DA metabolites homovanillic acid (HVA) and dihydroxyphenyl-acetic acid (DOPAC) [4,19]. Finally, columns were washed with 2 × 1.5 ml of 0.02 M ammonia and allowed to run dry. A 200-μl volume of the amine fraction was usually taken for the determination of the DA analogues by HPLC with amperometric detection. The remainder was used for the determination of DA, L-DOPA or noradrenaline (NA) [4,19].

Calibration curves

Stock solutions containing 100 μg/ml (referring to the free base) were prepared by weighing the appropriate amounts of the salts, which were dissolved in 0.01 M formic acid. These solutions, kept at 4°C, were stable in concentration for at least six months. Solutions of 1 μg/ml were made every month, while the lower concentrations were freshly prepared every day in water and the solution slightly acidified with formic acid.

Calibration curves were made by injecting the various standard solutions, measuring the peak heights in the chromatograms and plotting peak height (in cm or nA) against concentration or amount injected.

Precision was measured by repeatedly injecting the same solution and calculating the coefficient of variation [C.V.% = (S.D./$\bar{x}$) × 100] of the response.

Recovery

The recovery of the total assay was assessed by adding 25—100 μl of an appropriate standard solution to the glass tube with 0.5 ml of 0.1 M PCA and a piece of cerebellar or cortical tissue, and processing as described under Extraction procedure; 200 μl of the amine eluate were injected onto the column. The peak height was measured and compared to that of a standard solution. The recovery was calculated as follows:

recovery (%) = (h_{rec}/h_{st}) × $(C_{st} × 2.5/X)$ × 100

where X = ng added, h_{rec} = peak height of recovery, h_{st} = peak height of standard with concentration C_{st}, 2.5 = volume in ml of the collected fraction.

Recovery from serum or plasma was assessed identically: to 100 or 200 μl of serum or plasma the appropriate amounts were added and the above procedure was followed. The influence of the tissue weight was studied by varying this weight from 20 to 120 mg.

The precision of the recovery was determined by repeating the procedure 4—10 times and calculating the standard deviation (S.D.).

RESULTS AND DISCUSSION

Chromatography

Fig. 2 shows the retention behaviour of a range of DA analogues (Table I) on Nucleosil 5 C_{18} with methanol as organic modifier in pH 4.0 McIllvaine
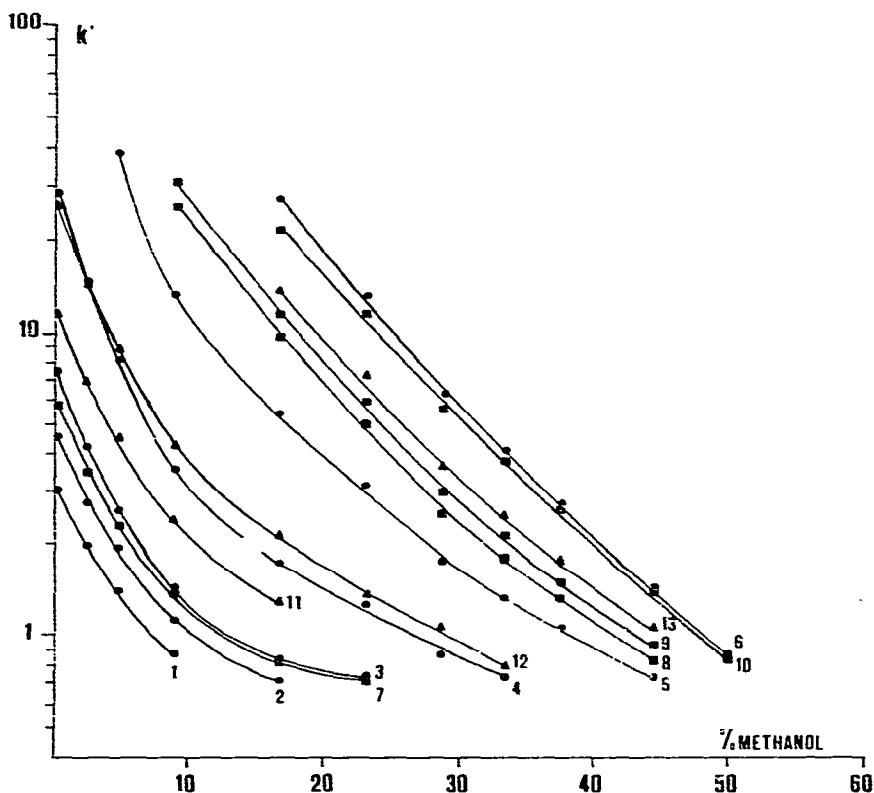
Fig. 2. Relationship between the percentage organic modifier methanol and the capacity factor (log k'). Experimental conditions: column, Nucleosil 5 C_{18}; eluent, pH 4 McIllvaine buffer; flow-rate, 1.0 ml/min; detector, rotating disc amperometric detector; oxidation potential, 700 mV; injected volume, 200 μl; concentration of the injected solutions, 200—1000 ng/ml. (●) Dopamine-derived catechols: 1 = DA, 2 = Epi, 3 = DiMe-DA, 4 = DiEt-DA, 5 = DiPr-DA, 6 = DiBu-DA. (■) 2-Aminotetralin-derived catechols: 7 = 6,7-ADTN, 8 = DiPr-6,7-ADTN, 9 = DiPr-5,6-ADTN, 10 = APO. (▲) O-Methyl derivatives: 11 = 3-MT, 12 = 7-O-Me-ADTN, 13 = 3-O-Me-DiPr-DA.

buffer. Similar curves were obtained using acetonitrile as organic modifier (results not shown). The relation between the percentage organic modifier and log k' is not linear, as was expected from the literature data [28,29]. The strongest curvature appeared at $k' < 2$ and about 10% organic modifier. For the remaining part of the curves a linear approximation may be adequate. Schoenmakers et al. [29] state that the curves are parallel with acetonitrile but divergent with methanol and tetrahydrofuran. However, the separation factors we obtained for several pairs of compounds were found to increase steadily with decreasing concentrations of methanol or acetonitrile, thus indicating divergent curves regardless of the organic modifier. No specific effects of acetonitrile were noted compared to methanol, except that APO is eluted faster with acetonitrile. The only difference between methanol and acetonitrile seems, therefore, to be that the same percentage acetonitrile reduced the retention by about 50%. As acetonitrile presented no advantages, further experiments were performed exclusively with methanol as organic modifier.

Essentially the same results were obtained with the Phenyl, RP-8 and RP-2 columns. The relation between percentage methanol and log k' is shown in Fig. 3 for two compounds, DA and DiPr-DA, on the four columns used. For the lipophilic compounds the Phenyl column causes the strongest retention and the RP-2 the weakest, while C_{18} and RP-8 do not differ very much from each other. However, for the hydrophilic compounds only the latter two columns gave any retention. The order of elution is generally the same, only APO is relatively more retained on the RP-8 column and less on the Phenyl column, compared to the C_{18} column. Thus, the separation factors for chemical substituents (3-OMe-DiPr-DA against DiPr-DA; DiBu-DA against DiPr-DA; DiPr-5,6-ADTN against DiPr-6,7-ADTN; etc.) are mostly comparable for all columns, with the above-mentioned two exceptions: (1) further "cyclization" of aminotetralins to APO results in a deviating chromatographic behaviour;
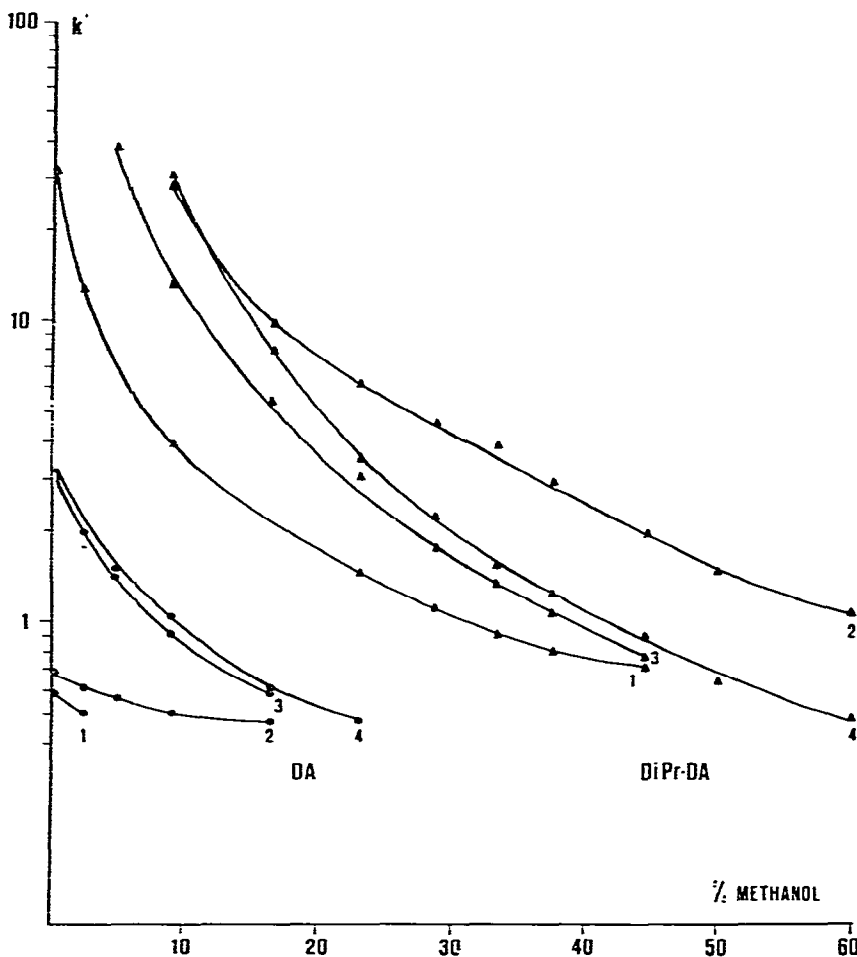

Fig. 3. Relationship between the percentage organic modifier (methanol) and the capacity factor (log k') on four different bonded-phase columns. 1 = LiChrosorb RP-2; 2 = Spherisorb S5 Phenyl; 3 = Nucleosil 5 C_{18}; 4 = LiChrosorb RP-8. (•)DA; (▲) DiPr-DA. Further experimental conditions as in Fig. 2.

(2) the RP-2 but especially the Phenyl column gives large separation factors between hydrophilic compounds like DA and weak lipophilic ones like DiMe-DA.

A McIllvaine buffer was chosen for the aqueous mobile phase as it is easily prepared and has a wide pH range (2.5—8.0). It has been shown that using this buffer with a properly chosen pH even strongly hydrophilic compounds such as NA and L-DOPA are sufficiently retained on Nucleosil 5 C_{18} without the need for a strong ion-pairing agent like octylsulphate in the mobile phase [4]. The addition of citrate to a phosphate buffer apparently increases the retention compared to a simple phosphate buffer as used by Molnar and Horváth [30]. Freed and Asmus [31] reported that citric acid, like trichloroacetic acid (TCA), increases retention of amines by ion-pair formation. The McIllvaine buffers seem to have an intermediate position between simple acids and ion-pairing agents like TCA and octylsulphate. No rapid column deterioration by citric acid [31] was observed. When used daily for biological samples, columns seemed to have a median lifetime of six months.

The chromatographic efficiency of the columns is strongly dependent on the structure of the test compounds. The efficiency of the column is substantially lower for amines than for acids like the natural metabolites of DA (DOPAC, HVA) and serotonin (5-hydroxyindoleacetic acid, 5-HIAA).

Typical plate numbers on the Nucleosil 5 C_{18} column are, for amines like DA and DiBu-DA with a k' of 4—6, n = 2500 (17,000 per m), and for acids like HVA and 5-HIAA with a k' of 4—6, n = 5000 (33,000 per m). The efficiencies obtained with the other packings were generally somewhat lower, while the tailing was more pronounced on the RP-2 column for all compounds and on the RP-8 column for APO and the O-methylated compounds.

The influence of the pH on the retention of acids is well known [30]. For amines an increase in retention with higher pH [30], accompanied by an increase in peak asymmetry [32], has been reported. We found by varying the pH from 4.0 to 6.5 that the more lipophilic amines are retained more at higher pH. The effect on the peak shape is detrimental, however.

Buffers more acidic than pH 4 may be used but they sometimes cause unstable baselines, especially when the Spectra-Physics pump was used.

From the above summarized results it was concluded that a system consisting of a column packed with Nucleosil 5 C_{18}, a pH 4 citrate—phosphate buffer as eluent and methanol as organic modifier represents a universal system for DA analogues. The separation of DiEt-DA, DiPr-DA, DiPr-6,7-ADTN, DiPr-5,6-ADTN and DiBu-DA on Nucleosil 5 C_{18} with 23.1% methanol in the eluent is shown in Fig. 4.

Detection

In Fig. 5 the sensitivity in nA/pmol for DA and some of its N,N-dialkylated analogues is shown as a function of the oxidation potential. The percentage methanol in the eluent was varied to keep k' at about 3 for all compounds. The sensitivity for DA is about twice that of the N,N-dialkylated analogues, while the O-methylated compound is only oxidized at potentials higher than 600 mV. Potentials higher than 700 mV were usually not applied for three reasons. Firstly, the offset current increases to sometimes unacceptable values;
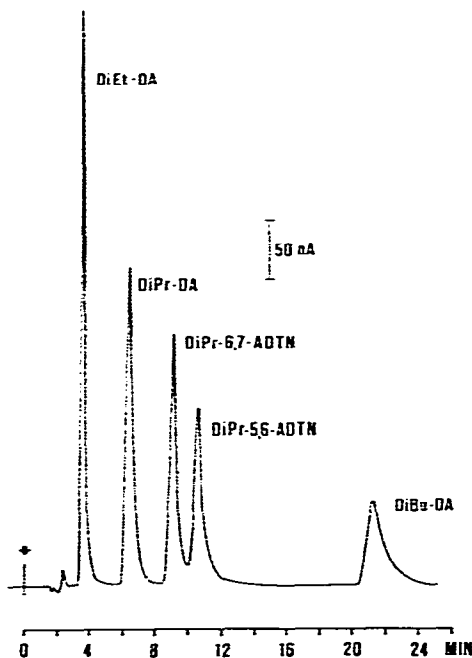
Fig. 4. Separation of five lipophilic DA analogues. Experimental conditions: column, Nucleosil 5 C₁ₛ; eluent, pH 4 McIllvaine buffer with 23.1% methanol; flow-rate, 1.0 ml/min; detector, rotating disc amperometric detector; oxidation potential, 700 mV; injected volume, 200 µl; potentiostat setting, 500 nA/V; concentrations of the injected solutions, 1000 ng/ml.

secondly, the baseline is not as stable as with lower potentials; and thirdly, the chance that endogenous compounds will interfere is increased.

The influence of the percentage methanol in the eluent was assessed by coupling the detector inlet directly to the injector outlet. The same difference between DA and its alkylated analogues exists, regardless of the methanol concentration. Also the sensitivities in this experiment were the same with 0 and 23% methanol. It may thus be concluded that the sensitivity is not affected by a relatively large concentration of methanol.

The influence of the pH on the sensitivity is hard to assess because of the serious disturbance of peak shapes at higher pH. However, at pH 5.5 there is no indication of an increased sensitivity for the N,N-dialkylated DA analogues, while at pH 6.5 the response is strongly decreased.

The distance of the electrode surface to the cell floor (where the inlet is located) is, depending on the cell geometry, an important parameter in the performance of the rotating disc electrode [4,33]. A very small distance (less than 0.2 mm) gives a high but fluctuating response, while the electrode surface is very easily damaged. Rotation causes a small increase in sensitivity. Increasing the distance results in a steadily decreasing sensitivity when the electrode is not rotating. For the rotating electrode an optimum can be found, where the sensitivity is at least as high as at a very small distance. At this optimum the difference in sensitivity between a rotating and stationary electrode is a factor of 5. The response at this height shows a better repro-
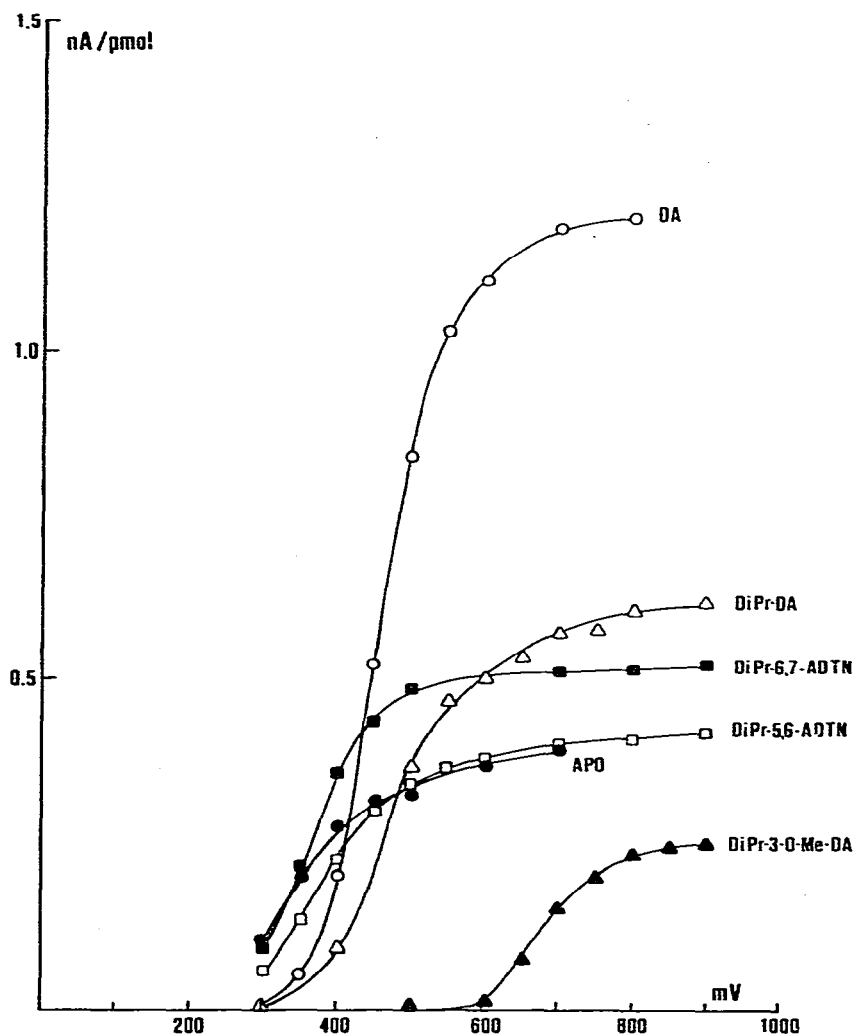
Fig. 5. Relationship between the response in nA/pmol and the oxidation potential in mV. Experimental conditions: column, Nucleosil 5 C_{18}; eluent, pH 4 McIllvaine buffer with varying percentages of methanol; flow-rate, 1.0 ml/min; detector, rotating disc amperometric detector; injected volume, 200 μl; concentrations of the injected solutions, 100—400 ng/ml. The percentage methanol was chosen so that the capacity factor was about 3 for all compounds.

ducibility, while the chance of damaging the electrode surface is minimal. Rotation provides a very thin diffusion layer at the electrode surface. Thus, the effective cell volume is very small compared to the total cell volume and peak broadening is minimal. All work reported here was carried out with the electrode at the optimal height (about 2.8 mm). Rotating the electrode only slightly increases the detector noise, and the signal-to-noise ratio is, like the sensitivity, increased by a factor of 5. This ratio is constant for a rotation speed up to 40 rps. Faster rotation results in a lower ratio. At a 500-mV oxidation potential the noise was usually 5—10 pA. The noise is mainly dependent

on the earthing of the rotating electrode. Further influences are rotation speed, cell geometry, condition of the carbon paste surface and the pH of the eluent. When a high sensitivity was used (0.5 or 1 nA/V) a Faraday cage around the detector was sometimes needed to prevent baseline instability.

The sensitivities reported by us (see also ref. 4) compare well to those reported in the literature (for example, ref. 34) for the more commonly used thin-layer detector cells. The rotating disc electrode is easy to work with and has a short stabilisation time; typically 15—30 min after applying the oxidation potential, a stable baseline was obtained at a setting of 1 nA full-scale. The carbon paste may be easily made in any laboratory and the electrode is readily repacked. The surface area of the electrode was about 40 mm^2.

Sample clean-up and recovery

N,N-Dialkylated DA and DA analogues of the DA and 2-aminotetralin series behave in much the same way on the small Sephadex G-10 columns as was reported for DA, NA, L-DOPA [4], 6,7- and 5,6-ADTN [22] and the O-methylated derivatives of DA (3-methoxytyramine, 3-MT [35]) and 6,7-ADTN (7-O-methyl-ADTN [23]). Thus the 2.0- or 2.5-ml fraction that is eluted with formic acid contains amines from DA to DiBu-DA with recoveries from 70 to 90%. As O-methylation tends to result in an earlier elution (for 3-MT see ref. 19), 3-O-Me-DiPr-DA has a lower recovery of about 60%. The preceding fraction, which indeed contains these compounds, is, however, less suited to sensitive determination as it gives a broad negative front.

Retention on Sephadex G-10 for amines is dependent on the formation of ion pairs between the amine and the perchlorate ion from the homogenization mixture [4]. This occurs at a low pH when the amine is protonated. The ion pair slowly dissociates and the amine is eluted. At a low pH the acidic (DA) metabolites are also retained. These acids, however, elute when the columns are washed with a phosphate solution of pH 7—8. Homogenisation in sulfuric acid, which is not an ion-pairing agent, or precipitation of the excess PCA in the supernatant, leaves the elution of the acids unaltered. The amines like DA [4,19] and DiPr-DA (unpublished results) are in this case much less retained and do not elute in the DA fraction.

These columns are easy to prepare and may be used for more than 6 months without any deterioration in the recovery of the amines. The recoveries are reproducible over a long period of time, representing 4—5 generations of columns.

Thus, these small Sephadex G-10 columns represent an easy and reliable way to prepare brain samples for the determination (with semi-automated fluorimetry or HPLC with amperometric detection) of biogenic amines and their acid metabolites [4,19,36], for the HPLC determination of their N-alkylated analogues and even for the gas chromatographic determination of the monohydroxy derivatives (Feenstra et al., unpublished results). It must be stressed, however, that introduction of a second phenyl group makes a compound unsuitable for this isolation procedure: APO is not eluted from Sephadex G-10, while 4-phenyl-6,7-ADTN is slowly eluted in all fractions. The increased lipophilicity may be responsible.

The recovery from serum or plasma is less, 55—70%, but with the same

reproducibility as from brain samples. It may be that more is coprecipitated with the proteins. From experiments in which the recovery of 50 ng of DiPr-DA was determined with and without added tissue it was found that about 10% DiPr-DA is lost with the various transfers before and during the column clean-up procedure and that another 10% is lost with the tissue homogenization. Increasing the tissue weight to over 100 mg did not increase this loss.

Fig. 6 shows chromatograms of serum (a, b) and cerebellar tissue (c, d) obtained from rats injected with 100 µmol/kg DiBu-DA and from control rats. No endogenous substances were found to interfere. This also applies to DiPr-DA, DiEt-DA, DiPr-5,6-ADTN, DiPr-6,7-ADTN and 3-O-Me-DiPr-DA. Determination of the more hydrophilic amines requires another pH (DA and NA [4]) or another eluent system (3-MT [35], and DiMe-6,7-ADTN in very low concentrations [37]) as endogenous compounds do interfere.

Quantitation

The present method can be used to determine concentrations of DA analogues in serum and brain tissue. The detection limit (the injected amount that gives a peak height which is four times the noise) for the N,N-dialkylated catecholamines was found to be 25—50 pg per injection from a standard solution and 40—100 pg from a biological sample. Methoxylated compounds have higher detection limits (50—100 pg in a standard solution and 100—200 pg in a biological sample for 3-O-Me-DiPr-DA), because the sensitivity is less (Fig. 5) and the baseline is less stable with the higher potential setting.

The calibration curves for all the compounds are linear from the detection limit at a setting of 1 nA/V to about 500 ng at the maximal attenuation of 500 nA/V. Table II lists results from a calibration and recovery experiment for DiPr-DA and 3-O-Me-DiPr-DA, in which 1, 10 and 100 ng were added to pieces of cerebellar tissue; a 2.0-ml amine fraction was collected, of which 200 µl were injected. Standards were diluted to the same extent with 0.01 M formic acid. Linearity, recovery and precision were excellent for DiPr-DA and acceptable for 3-O-Me-DiPr-DA. It might be argued that an even better precision would be obtained if an internal standard was used. To test this we determined the reproducibility of the total procedure with and without tissue. DiPr-6,7-ADTN (50 ng) was added as internal standard to DiPr-DA (50 ng). The concentration of DiPr-DA was calculated with and without the use of the internal standard. The results showed that the reproducibility is not affected by the use of an internal standard; the coefficients of variation were in both cases 4.4% (with added tissue) and 4.0% (without tissue), respectively. Therefore we feel that the use of an internal standard is not indicated.

Fig. 7 shows the time—course of serum and brain concentrations of DiPr-DA after intraperitoneal injections of 100 µmol/kg to female Wistar rats of about 200 g. It is clear that the compound rapidly achieved its maximum brain concentration, but only remained a short time in the brain. This short duration is to a large extent explained by the metabolic deactivation by the enzyme catechol-O-methyltransferase. A more detailed account of the brain concentrations of DA analogues will be presented elsewhere.

TABLE II

CALIBRATION AND RECOVERY OF DiPr-DA AND 3-OMe-DiPr-DA

Calibration: both compounds were added in the stated amounts to 2.0 ml of 0.01 M formic acid. Recovery: both compounds were added in the stated amounts to a piece of cerebellar tissue, which was treated as described under Extraction procedure. From the 2-ml fractions obtained, 200 μl were injected onto the Nucleosil 5 C_{18} column. Eluent: pH 4 McIlvaine buffer—20 and 28% methanol. Detector potential:500 and 700 mV (for DiPr-DA and 3-O-Me-DiPr-DA, respectively). Potentiostat: 10 or 1 nA/V. Response was calculated from the peak height. Recovery was calculated as the response of the extracted sample divided by the response of the standard sample. For all groups: $n = 5$. Correlation and slope were calculated after log—log conversion of all individual data.

| Compound	Added (ng)	Injected (ng)	Standard		Recovery from brain tissue		
			Response (nA)	C.V. (%)	Response (nA)	C.V. (%)	Recovery (%)
N,N-Dipropyl-dopamine	100	10	12.0	2.4	9.6	4.7	80.0
	10	1	1.2	3.5	0.97	4.7	80.8
	1	0.1	0.12	3.1	0.095	3.6	79.2
Correlation coefficient (r^2)			0.999		0.999		
Log—log slope			1.004		1.005		
3-O-Methyl-N,N-dipropyl-dopamine	100	10	4.95	1.0	3.0	12.7	60.6
	10	1	0.46	8.2	0.3	5.8	65.2
	1	0.1	0.055	24.9	Not detectable		
Correlation coefficient (r^2)			0.994		0.994		
Log—log slope			0.982		0.986		
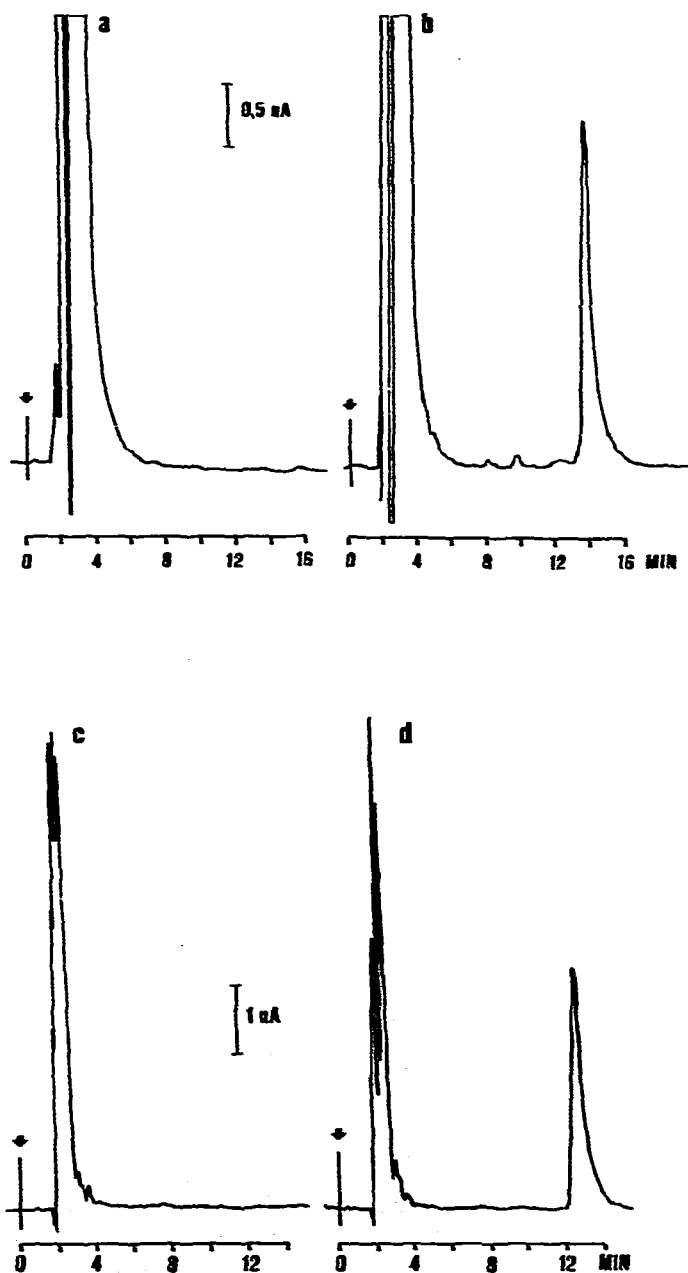
Fig. 6. Detection of DiBu-DA in rat brain and serum. (a) 500 μl of serum from a control rat. (b) 500 μl of serum from a rat that was 10 min previously injected with 100 μmol/kg DiBu-DA. Calculated concentration = 120 ng/ml (injected 4.8 ng). (c) 40.0 mg of cerebellar tissue of a control rat. (d) 40.7 mg of cerebellar tissue of a rat that had been injected 20 min previously with 100 μmol/kg DiBu-DA. Calculated concentration = 1.92 μg/g (injected 6.3 ng). Experimental conditions: column, Nucleosil 5 C_{18}; eluent, McIllvaine buffer pH 4 with 28.6% methanol; flow-rate, 1.0 ml/min; detector, rotating disc amperometric detector; oxidation potential, 500 mV; injected volume, 200 μl.
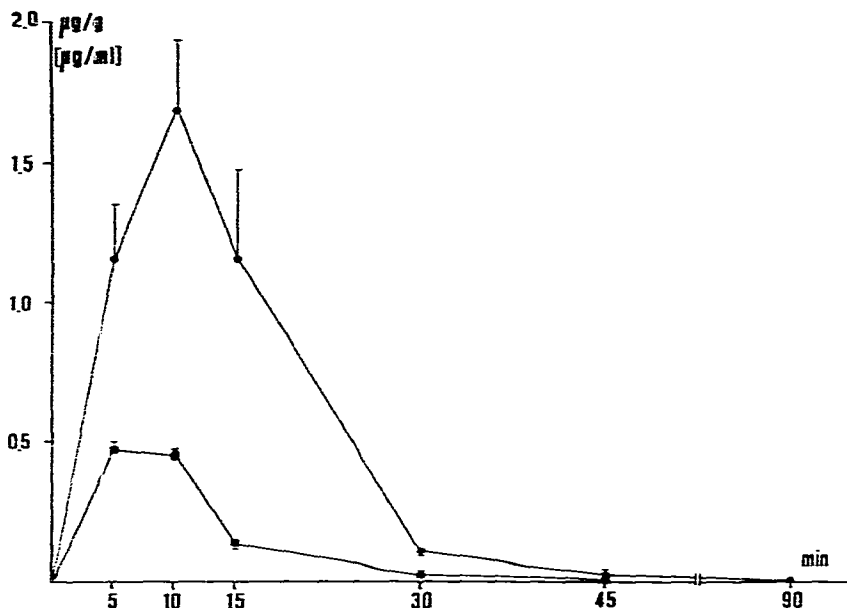
Fig. 7. Concentrations of DiPr-DA in striatum (●) and serum (■) of rats injected intraperi-toneally with 100 μmol/kg. Experimental conditions: as in Fig. 6 but 20.0% methanol. The values presented are the means (± S.E.M.) of four rats. The concentrations were corrected for recoveries which were 83.4% (± 7.2% S.D.) for striatum ($n = 4$) and 67.5% (± 3.4% S.D.) for serum ($n = 4$).

CONCLUSION

Up until now the only reported method of determination of N-alkylated DA analogues other than APO was a radioenzymatic assay for DiPr-DA [8], which was, however, only used for the measurement of blood levels of this compound. The present method, with its use of a short and easy sample clean-up procedure on Sephadex G-10 columns, and a selective detection with a relatively low oxidation potential of 500 mV after separation on a reversed-phase HPLC column, provides a simple, sensitive and reliable way to detect and quantify DA analogues in serum and small pieces of brain tissue of experimental animals. Furthermore, the method is very versatile as only slight variations in the experimental conditions, such as the pH and the percentage methanol in the eluent or the oxidation potential, also allow the determination of the methoxylated derivatives of the DA analogues as well as a wide range of endogenous catecholamines and metabolites. The effects of the DA analogues on the brain concentrations of DA and its metabolites can be used as a method to assess the dopaminergic potency of the analogues [38].

Thus, the present method makes it possible to determine in one and the same piece of brain tissue the concentration of the pharmacological agent and the effect it exerts on the dopaminergic system.

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