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ASSAY PROCEDURES FOR THE DETERMINATION OF BIOGENIC AMINES AND THEIR METABOLITES IN RAT HYPOTHALAMUS USING ION-PAIRING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R.B. TAYLOR*, R. REID, K.E. KENDLE, C. GEDDES and P.F. CURLE

School of Pharmacy, Robert Gordon's Institute of Technology, Schoolhill, Aberdeen AB9 1FR (U.K.)

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SUMMARY

Procedures are described for the simultaneous determination, by high-performance liquid chromatography of adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine using laurylsulphate as pairing ion and for the separate simultaneous determination of vanillylmandelic acid, dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylethylene glycol, 5-hydroxyindoleacetic acid and homovanillic acid using tetraethylammonium as pairing ion. Sample preparation consists of protein precipitation only and octadecylsilane coated silica is used for both sets of determinations as is electrochemical detection.

The chromatographic basis of each separation is discussed in the light of modern ideas of ion exchange and desolvation to enable the method to be modified chromatographically if other compounds are to be resolved or the assay method modified. The quantitative aspects of the methods are detailed and applied to amine and metabolite levels in rat hypothalamus. The values determined together with their sample variation and estimated limits of detection are quoted.

INTRODUCTION

The determination of adrenaline (A), noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5HT) in brain and other biological matrices has been the subject of several reports using pretreatment stages involving alumina adsorption to isolate the amines from predominantly acidic metabolites [1—4]. In these cases, chromatographic retention and separation have been achieved using aqueous methanol eluents with or without the addition of hydrophobic pairing ion, usually octylsulphate. The only separation and simultaneous quantitaton of the recognised metabolites of the above compounds vanillyl-

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mandelic acid (VMA), dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) have been achieved by gradient elution in buffered aqueous methanol after extraction [5].

While numerous other reports have appeared in the literature describing methods for the determination of particular metabolites [6, 7] or amines [8] or unique combinations of amine and metabolites [9—14], these are open to criticism on the grounds that resolution is often inadequate for complete specificity of determination [15, 16].

More seriously, in many reported methods other amines and/or metabolites which have not been removed by any pretreatment step are not accounted for in the chromatographic separation. Such criticism is particularly relevant in view of recent methods where cerebrospinal fluid or brain tissue homogenate has been injected directly onto the chromatographic column subsequent only to protein precipitation [17, 18]. Such samples must necessarily contain all amines and metabolites and any specific estimation by chromatography must at least justify why the non-quantitated species do not interfere. Certain reports do in fact indicate doubts concerning the complete specificity of chromatography [10, 19].

The present work attempts to overcome these shortcomings and describes two isocratic separations, one suitable for the determination of the above amines in presence of the metabolites, the other for the determination of acidic and neutral metabolites in the presence of the parent amines. The biological matrix used is rat hypothalamus subjected to protein precipitation pretreatment only. Preliminary chromatographic retention data are obtained using ultraviolet detection and quantitation at the biological level is by electrochemical detection (ED). The compounds listed are chosen since they are currently believed to be the main biogenic amines and metabolites involved in central neurotransmission [20, 21].

EXPERIMENTAL

Equipment

Chromatographic measurements were carried out using a Waters Assoc. M 6000 A pump and M 440 fixed-wavelength (254 nm) or Bioanalytical Systems LC4 detectors. Columns were 200 × 4.6 mm slurry packed using 5-μm ODS Hypersil (Shandon Laboratories). The electrochemical detector was fitted with a polished glassy carbon electrode and operated at an applied potential of 0.70 V relative to a silver/silver chloride reference electrode. The column to detector cell fittings were modified to reduce dead volume.

Chemicals

DOPAC, NA (bitartrate), 5HIAA, HVA and VMA were obtained from Sigma. 5HT (sulphate) and DA (hydrochloride) were obtained from BDH and A (hydrogen tartrate) from Koch-Light. MHPG (piperazine salt) was obtained from Aldrich as were the pairing ions tetraethylammonium (TEA) and tetrabutylammonium (TBA) as the bromides. Laurylsulphate (SLS) as the sodium salt was obtained from Fisons. Acetonitrile was obtained from Rathburn

Chemicals and water was purified using a Milli Q system. All other chemicals used were of AnalaR or equivalent grade.

Sample preparation

Mature male rats of the Sprague—Dawley strain from our own breeding colonies were killed by cervical dislocation. The hypothalami were removed and placed in a vessel in an ice bath within 2 min of death. The tissues were weighed and homogenised by hand using a 1-ml glass Potter homogeniser within 1 h of removal from the animal. Approximately 0.1-g tissue samples were homogenised in 500 μl of 0.4 *M* perchloric acid. After centrifuging the homogenate at 3000 *g* for 30 min the supernatant (20 or 50 μl) was injected via a Rheodyne 7125 valve.

THEORETICAL BASIS OF SEPARATIONS

Previous high-performance liquid chromatographic (HPLC) determinations of these amine metabolites have been carried out in buffer solutions at pH values low enough to suppress ionisation of the predominantly acid metabolites. Retention in these cases is on the basis of varying hydrophobicity of essentially neutral solutes. In such cases the amines, being fully protonated, should elute rapidly. In some investigations pH is manipulated so as to achieve retention of certain of the amines as well as metabolites [15]. The corresponding methodology cannot be applied to the determination of bases alone since at pH values high enough to deprotonate the bases completely, catecholamines are chemically unstable [22] and in addition, the bonded hydrocarbon stationary phase is degraded [23]. The strategy in such cases has been to include a hydrophobic pairing ion such as octylsulphonate in a low pH buffer in order to retain bases by ion pairing and also to retain acid and neutral metabolites in the same system by ion suppression [24]. This latter procedure can result in crowded chromatograms with resolutions often quite inadequate for quantitation. Such methods are characterised by the use of very low solvent pairing ion concentrations.

Investigations on the mechanism of ion-pairing chromatography have shown that the dependence of capacity factors of solutes on solvent pairing ion concentration is complex [25] and that very often such a dependence goes through a maximum. Such behaviour has been explained on the basis of an ion-exchange—desolvation mechanism [26, 27]. Retention of a solute on a C-18 coated surface containing adsorbed pairing ion of charge opposite to that of the solute is described as occurring due to electrostatic binding coupled with desolvation of the neutralised solute on the available C-18 surface; i.e. on surface unoccupied by adsorbed pairing ion. Thus, if the adsorbed pairing-ion concentration is high due either to a high solvent concentration or to the use of a very strongly adsorbed pairing ion, retention of both charged and neutral species will actually be reduced due to the unavailability of free C-18 surface for desolvation.

The mixtures of basic biogenic amines and their predominantly acidic metabolites constitute an ideal system to which the above ion exchange—desolvation ideas may be applied for the purpose of obtaining improved

specificity in the assay of these compounds. At low pH the amines may be retained and resolved by suitable choice of anionic pairing ion at an appropriate concentration while the retention of acid and neutral metabolites may be minimized. Similarly at pH 7 the acidic compounds being appreciably ionised can be retained by use of a suitable cationic pairing ion while minimising the retention of the bases whether or not they are completely uncharged.

In principle, therefore, it should be possible to optimise the effects of increasing and decreasing retention of the different classes of solutes by choice and concentration of added pairing ion to achieve the required separations.

RESULTS AND DISCUSSION

Separation of amines

Fig. 1 shows the effect of SLS concentration in the solvent on the capacity factor (k') for the nine solutes studied. The amines show the predicted maxima

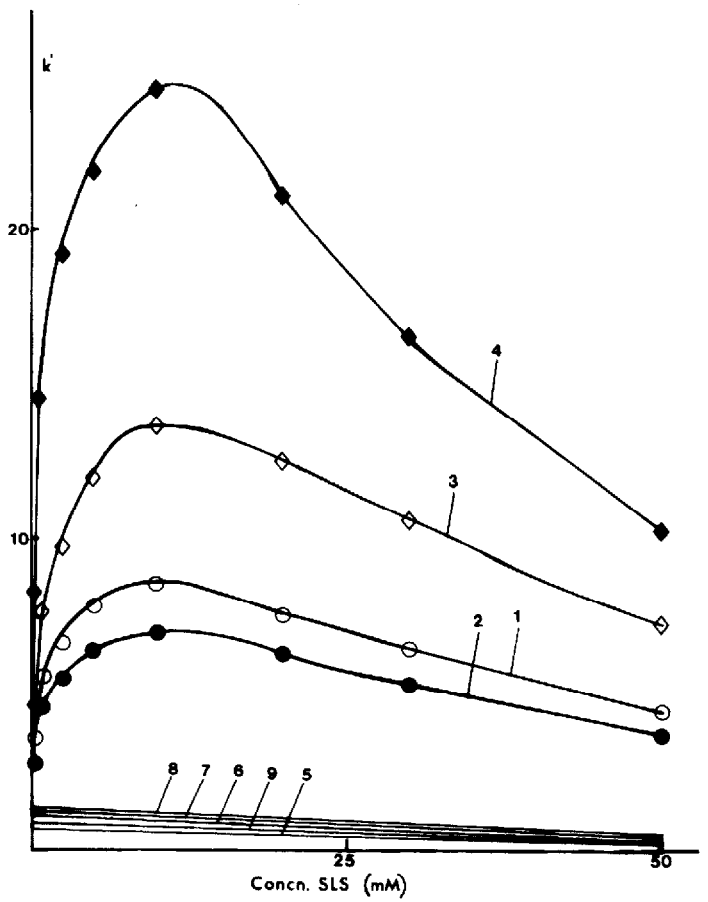

Fig. 1. Variation of capacity factor k' with SLS concentration for the nine amines and metabolites studied. Column 200 × 4.6 mm ODS Hypersil (5 μm); solvent 10 m*M* disodium hydrogen phosphate, pH 2 buffer containing 25% acetonitrile and 0.5 m*M* EDTA. Compound identification: 1 = A; 2 = NA; 3 = DA; 4 = 5HT; 5 = VMA; 6 = DOPAC; 7 = MHPG; 8 = 5HIAA; 9 = HVA.

while the metabolites show very low retentions which are further reduced by increasing SLS concentration. This pairing ion was chosen because of its ready availability and cheapness. Although in purely aqueous solvent it will produce very large capacity factors for bases [27], this is overcome by the inclusion of organic modifier which, in turn, will aid the rapid elution of neutralised acidic metabolites and endogenous materials from biological samples. Fig. 1 shows that pairing-ion concentration will dictate the degree of resolution obtained. While low concentrations (3—5 m*M*) can provide adequate resolution higher concentrations (10—50 m*M*) provide a less critical system with respect to pairing-ion concentration, avoid the problem of long equilibration times [28] and further reduce the retention of neutral components.

Representative separations of the major amines in a protein-precipitated homogenate are shown in Fig. 2a. Fig. 2b shows the separation of a standard mixture of amines together with their metabolites while Fig. 2c showing the separation of a spiked homogenate extract verifies the identities of the peaks shown. Comparison of Fig. 2a and b indicates the absence of both endogenous compounds and metabolite interference in the determination of amines using this system. The critical resolution was found to be the A/NA pair and acetonitrile afforded better selectivity than methanol which produced unreasonably long retention times for 5HT to achieve complete A/NA separation.

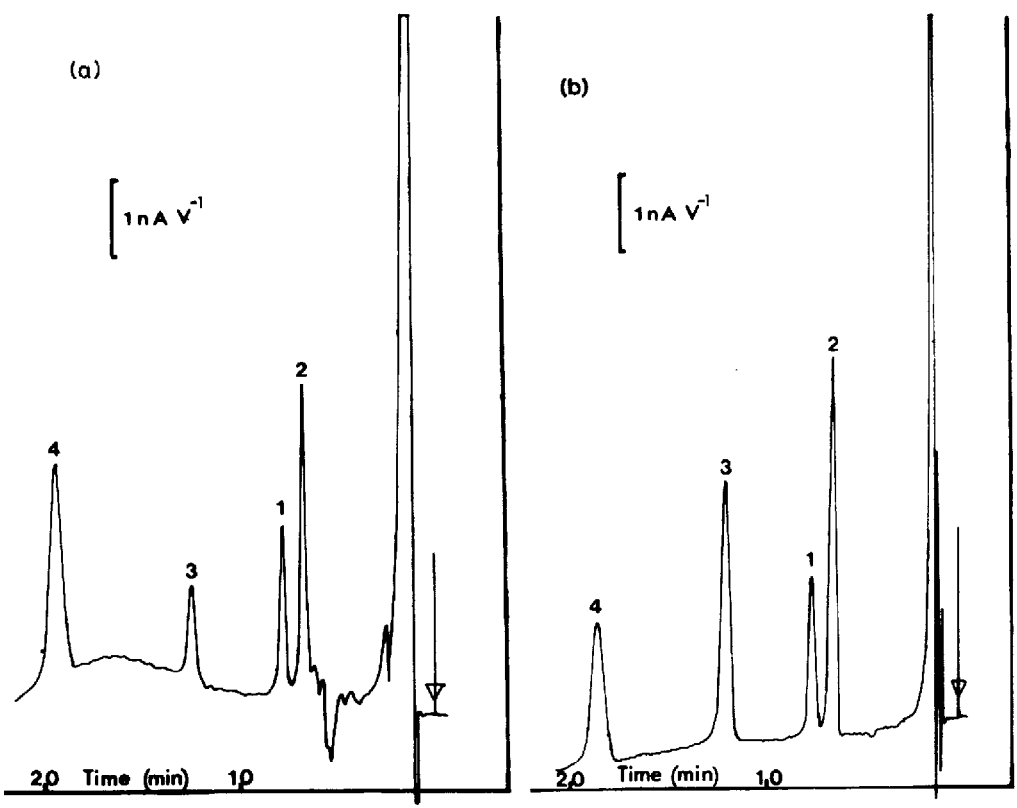

Fig. 2.

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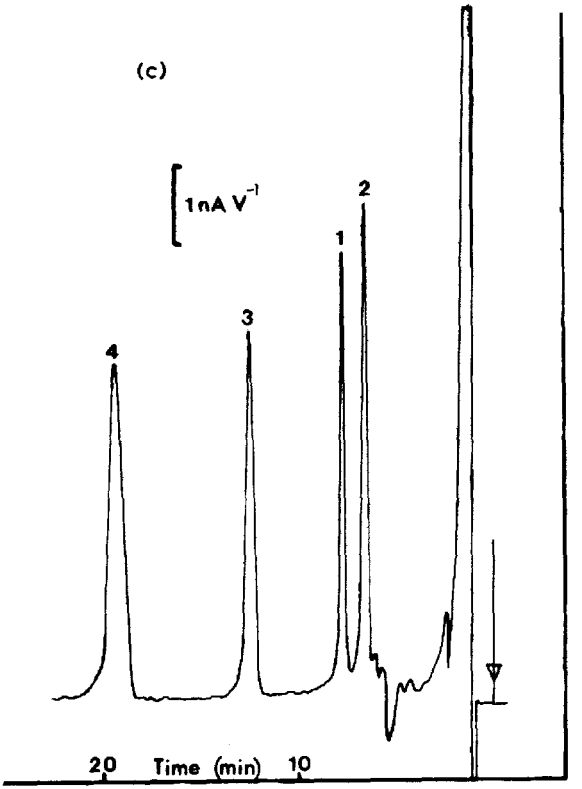


Fig. 2. Representative chromatograms showing separation of the major amines. (a) Supernatant from protein-precipitated tissue homogenate; (b) all amines and metabolites in aqueous solution; (c) tissue homogenate spiked with added metabolites. Conditions and compound identification as in Fig. 1 with SLS concentration 20 m*M*.

Separation of acid and neutral metabolites

To achieve this separation and concomitant minimal retention of amines it was found that the choice of pairing ion was critical. Very hydrophobic cationic pairing ions such as tetrabutylammonium or cetyltrimethylammonium while providing adequate resolution of the acidic species, reduced the retention of the neutral MHPG metabolite too rapidly with increasing pairing-ion concentration. The metabolite because of its highly hydroxylated structure and correspondingly low hydrophobicity was, in fact, less retained on most systems than the basic 5HT at pH 7.

Tetraethylammonium pairing ion proved to be the most suitable choice. Fig. 3 shows the variation of capacity factor, k', with TEA concentration for all solutes studied. At low TEA concentrations the neutral MHPG is highly retained as is 5HT while retention of the acidic metabolites is inadequate for reliable determination in biological matrices. Since TEA is a relatively weakly adsorbed pairing ion [26], as the TEA concentration is increased the retention of MHPG is only gradually reduced. The accumulation of charge on the C18 surface, however, decreases the retention of the 5HT markedly while having the expected ion pairing retention effect on the acidic anions. In this system the optimal concentration of TEA used was considered to be 30 m*M* and at this

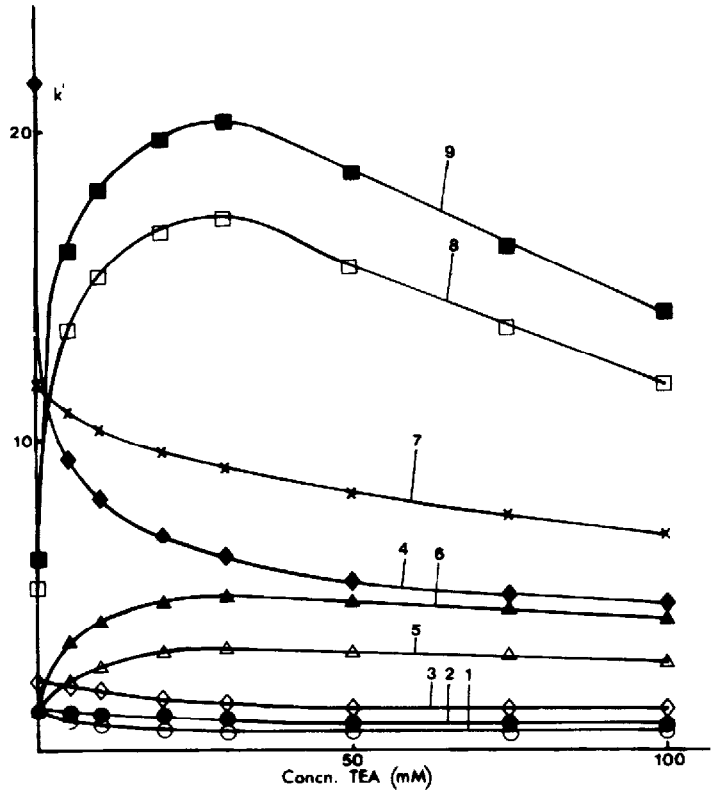
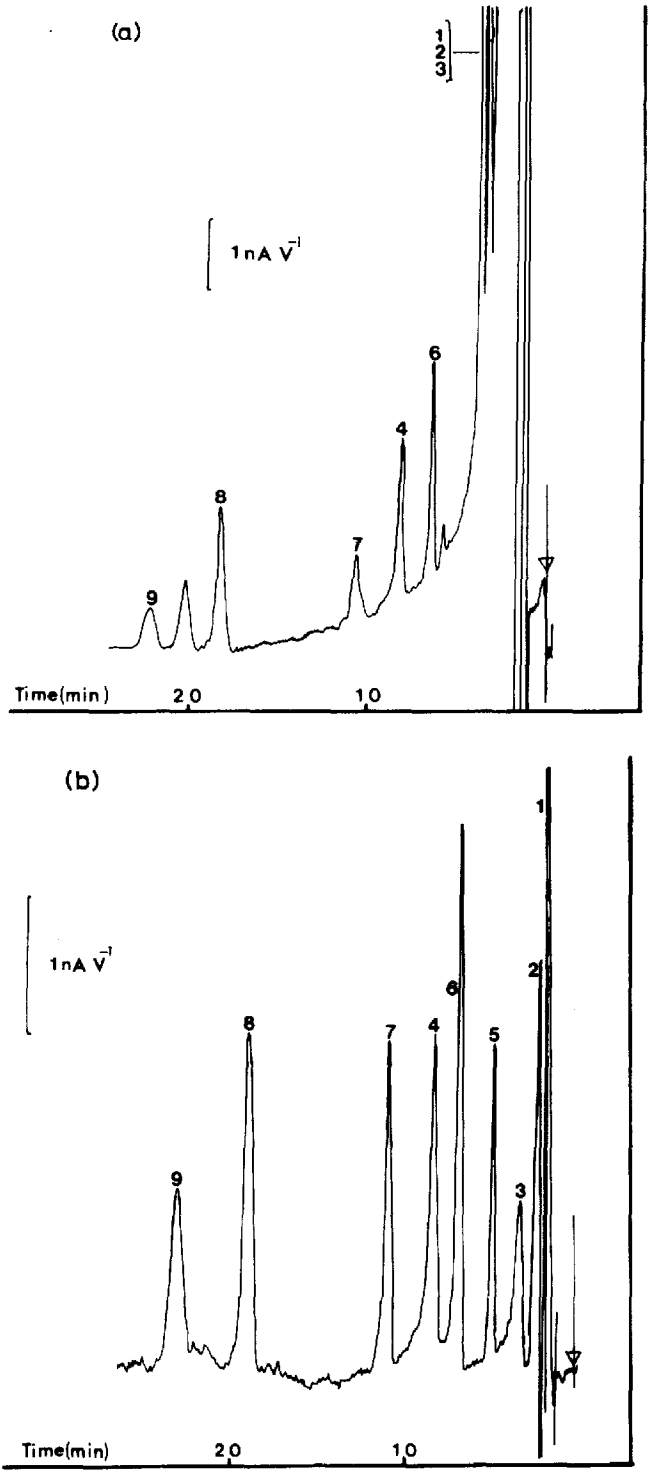


Fig. 3. Variation of the capacity factor k' with TEA concentration for the nine solutes studied. Column 200 × 4.6 mm ODS Hypersil (5 μm); solvent 10 mM disodium hydrogenphosphate, pH 7 buffer 0.5 mM in EDTA. Compound identification as in Fig. 1.

concentration NA, A and DA were rapidly eluted while 5HT in spite of its predominantly basic character was retained but resolved from the metabolite species. It was not possible to include organic modifier in this system for the purpose of reducing the peak due to endogenous materials.

Fig. 4a shows a representative chromatogram of tissue extract run on this solvent system. The signal due to endogenous material is much larger than in the case of the amine system but resolution is adequate. Fig. 4b is a chromatogram of a standard mixture of amines and metabolites and verifies that the amines other than 5HT are eluted with the residual endogenous compounds. Fig. 4a indicates an absence of VMA in the sample and this was found generally to be the case for other hypothalamus samples measured. Fig. 4c, a chromatogram of tissue spiked with all compounds, confirms the identities of the various peaks and the absence of VMA in the sample. In this solvent system few unidentified peaks were evident at the sensitivity required for the major metabolites. In Fig. 4a and c only one unidentified peak is noted between 5HIAA and HVA while in other brain samples assayed an additional peak was, on occasion, recorded after HVA. Resolution was adequate in all cases for quantitation of the specified metabolites.

It is emphasised that the two solvent systems described are not unique and


(a)
1
2
3
1nA V⁻¹
6
4
8
7
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Time(min)
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1nA V⁻¹
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Fig. 4.

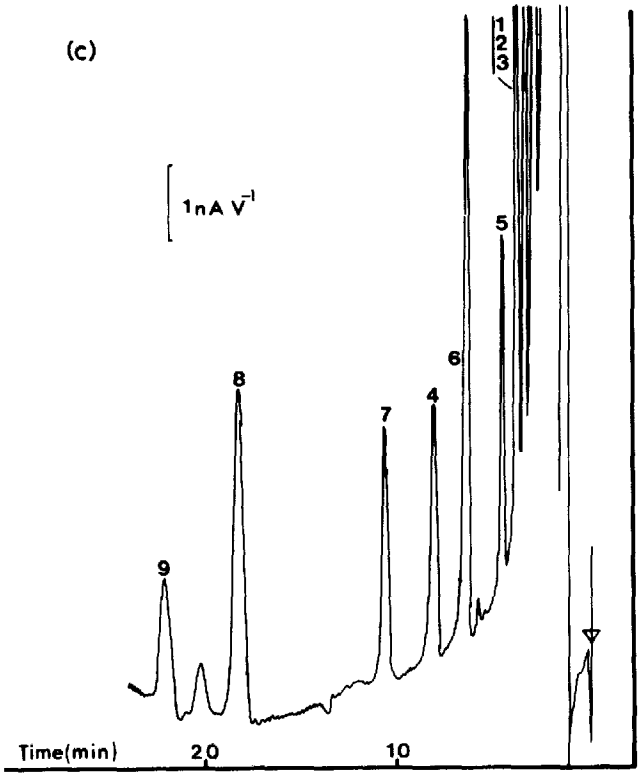

Fig. 4. Representative chromatograms showing separation of the amine metabolites and 5HT. (a) Supernatant from protein-precipitated tissue homogenate; (b) all amines and metabolites in aqueous solution; (c) tissue supernatant spiked with added metabolites. Conditions as in Fig. 3 with TEA concentration 30 m*M*. Compound identification as given in Fig. 1.

have been chosen as optimum for the particular purpose of determining the specified compounds. For other investigations it would be possible to alter retention times and order of elution by choice of different pairing ions and concentrations bearing in mind the dual retention characteristics of ion exchange and hydrophobicity.

Quantitation

For both amines and metabolites the standard addition method was applied to a pooled tissue sample to verify the linearity of the chromatographic response. Fig. 5a and b demonstrate the resultant plots, and confirm the linearity of response and verify the specificity of the solute peaks. The constants of the regression lines are listed in Table I. Concentrations of the endogenous catecholamines and metabolites are not quoted since different pooled samples were employed but the slopes of the regression lines indicate the relative sensitivities of the method for the different solutes. It is also seen from Table I that good agreement is obtained between experimental intercepts in Fig. 5a and b and those obtained by regression. The somewhat lower sensitivities apparent compared with other workers using similar detection systems [29] may be a consequence of the lower applied voltage and/or the

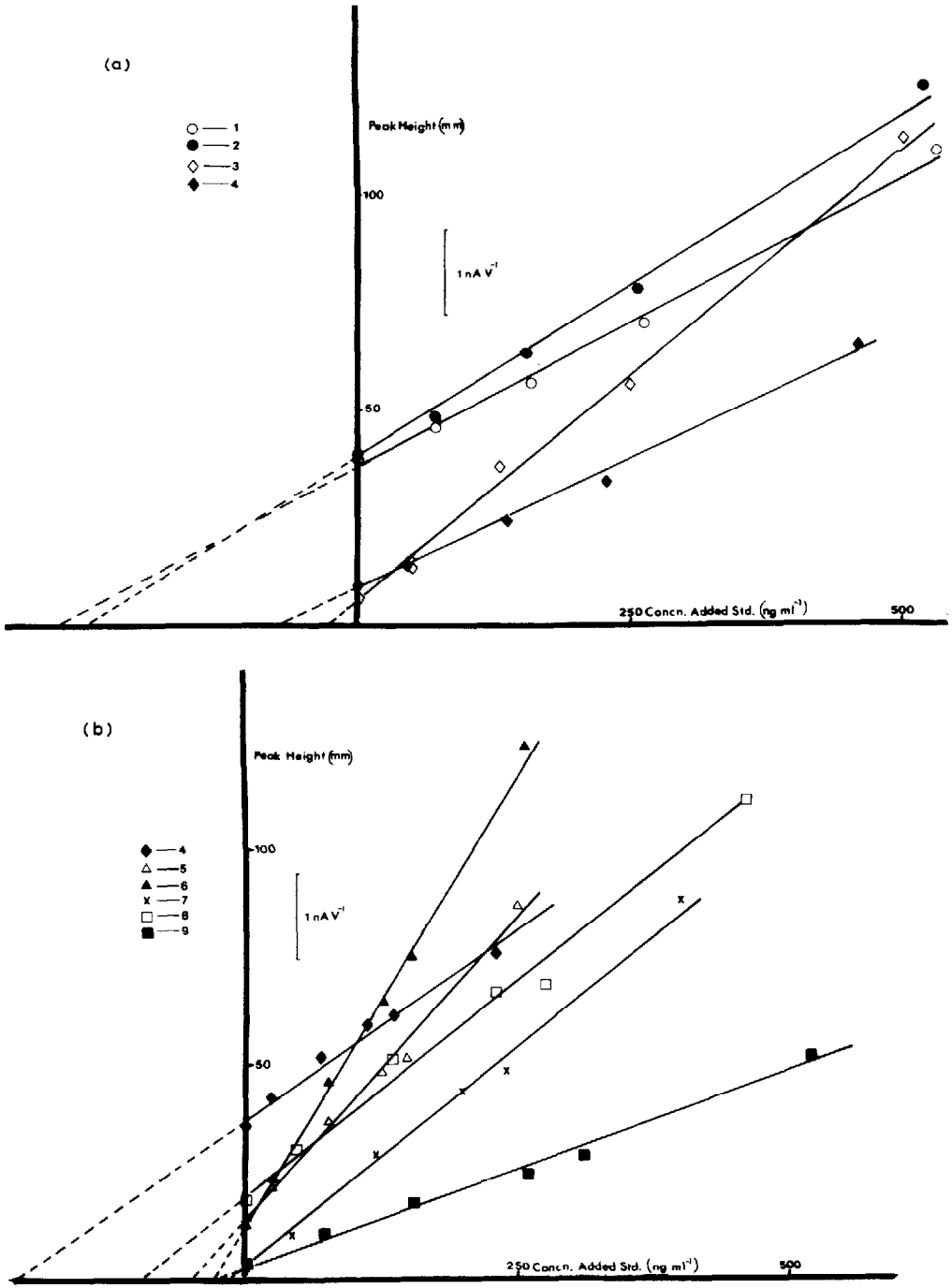

Fig. 5. Plots showing the variation of peak heights on addition of added standards to pooled protein-precipitated tissue homogenate. (a) Amines added, results from SLS system; (b) metabolites added, results from TEA system. Compound identification as in Fig. 1.

TABLE I

CONSTANTS OF THE REGRESSION LINES OBTAINED BY STANDARD ADDITION OF AMINES AND METABOLITES TO TISSUE SAMPLES

Compound	Constants of regression line					
	Slope	S.D. of slope	Intercept (regression)	Intercept (experimental)	S.D. of intercept	Correlation coefficient, r^2
A	0.125	$3.79 \cdot 10^{-3}$	37.3	38.4	1.04	0.998
NA	0.154	$3.32 \cdot 10^{-3}$	39.2	39.8	0.898	0.999
DA	0.205	$4.49 \cdot 10^{-3}$	5.02	6.3	1.16	0.999
VMA	0.282	$1.49 \cdot 10^{-3}$	13.7	14	1.99	0.994
DOPAC	0.433	$1.14 \cdot 10^{-3}$	11.4	12.4	1.54	0.998
MHPG	0.212	$8.19 \cdot 10^{-3}$	2.02	3.2	1.74	0.997
5HIAA	0.194	$1.13 \cdot 10^{-3}$	19.7	17.8	2.77	0.993
HVA	0.089	$5.43 \cdot 10^{-3}$	3.12	2.8	1.50	0.992
5HT bases	0.120	$6.11 \cdot 10^{-3}$	7.91	9.0	1.46	0.996
5HT acids	0.167	$9.58 \cdot 10^{-3}$	38.1	35.8	1.17	0.993

chromatographic solvent used. Table II shows results obtained on individual hypothalamus samples obtained by direct comparison of sample peaks produced with those of a set of aqueous standards. The 5HT values shown represent the means of the two values determined on the different solvent systems and the MHPG values refer to the unconjugated glycol. Also included in Table II are literature values for the concentrations of these compounds in rat hypothalamus.

The values obtained in the present study are in general agreement with those reported in the literature. None of the reported investigations, however, gave data for all of the compounds used in the present study. The apparent differences between present and literature values are probably a reflection of differences in technique. The present method involves minimal sample preparation with consequent minimal opportunity for losses. This may explain why noradrenaline and 5HT levels are similar to or greater than the highest levels previously reported. In the case of 5HT preliminary studies with aqueous standards showed its chemical instability so the high value in the present study may also be a reflection of the speed of sample preparation. Although HVA was reported in the literature it was not detected in the hypothalamic homogenates used for quantitation. It was, however, present in the homogenate used to establish retention times as indicated in Fig. 4a and c. The tissue for these experiments was isolated by different workers and it is likely therefore that the presence or absence of HVA may depend on minor variations in the size of the region isolated as hypothalamus. The limits of detection for the solutes by the present method determined in aqueous solution are also included in Table II. The chromatographic precision of the method (R.S.D. = 2.0%, $n = 10$) was considerably below the biological variation shown in Table II.

TABLE II

RESULTS OF AMINE AND METABOLITE DETERMINATIONS ON SIX TISSUE SAMPLES BY THE PRESENT METHOD TOGETHER WITH LITERATURE VALUES FOR CERTAIN COMPOUNDS IN THE MALE RAT HYPOTHALAMUS

Investigation method	Concentration ng/g wet tissue (R.S.D.)								
	A	NA	DA	5HT	VMA	DOPAC	MHPG	5HIAA	HVA
This investigation	78.3	3066	502	1812	20	100	97	409	25
Rat hypothalamus (n=6)	(51)	(27)	(12)	(17)		(17)	(14)	(10)	
HPLC–ED									
Limit of detection (ng/ml)	33	26	21	26	6.6	9.4	8.5	11	25
HPLC–ED [8]	—	808	150	—	—	—	—	—	—
HPLC–ED [30]	—	2780	596	1098	—	—	—	—	—
Gas chromatography–	—	2418	569	956	—	—	—	565	95
mass spectrometry	—	1740	488	1089	—	160	92	560	—
[31]	—	1707	359	741	—	—	—	414	116
	—	2327	465	931	—	—	—	483	111
HPLC–ED [32]	64	2080	410	—	—	46	—	—	35
	58								
	66								

CONCLUSION

The reported methods rely on minimum pretreatment of tissue samples with consequently least opportunity for sample loss. The chromatographic systems represent an advance on current literature systems since they provide improved resolution over all but gradient elution methods for all the accepted metabolites or amines and they also accommodate the presence of all of these compounds. The disadvantage of the two-solvent column system is believed to be more than compensated by the completeness of metabolite detection, the high capacity factors and improved resolution. The apparently lower sensitivities achieved are adequate for quantitation with the possible exception of VMA but could be improved by modification to column geometry and injection volume if required. The identities of the unknown compounds will be the subject of a continuing investigation as will determination of amine turnover rates for which purpose the assay methods described were designed.

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