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Note

Alkyl boronates as catechol-specific mobile phase pairing agents

Application to high-performance liquid chromatographic analysis of amines, precursors and metabolites in brain tissue

MICHAEL H. JOSEPH*

Division of Psychiatry, MRC Clinical Research Centre, Watford Road, Harrow HA1 3UJ (U.K.)

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Reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection provides a simple and sensitive approach to the analysis of the principal biogenic amines, noradrenaline (NA), dopamine (DA) and serotonin (5HT), and their precursors and metabolites. In principle all of them can be separated and determined in a single chromatographic run, using suitable eluting buffer conditions with respect to pH, proportion of organic modifier, and nature and concentration of buffer anion [1–3]. Buffer pH can be used to control the retention of acidic monoamine metabolites, since their pK_a values lie within the range compatible with reversed-phase packings (pH 2–8) [4, 5]. The parent amines, however, are positively charged throughout this range; their retentions can then be controlled by addition of ion-pairing agents (usually alkyl sulphates or sulphonates such as sodium octyl sulphate) to the running buffer (see ref. 6).

In practice, it is often difficult to combine sufficient retention of noradrenaline (one of the most hydrophilic species of interest), to separate it from the unretained peak, with an acceptably short overall run time, because of unduly long retention of 5HT. Preliminary spearation of catecholamines (NA, DA and other catechols) (see for example ref. 7) using alumina is time-consuming and vitiates the advantages of single-chromatogram determination of many metabolites. Alkyl boronates are used as derivatisation reagents for gas-

*Present address: Departments of Psychology and Biochemistry, Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF, U.K.

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liquid chromatographic analysis of molecules with hydroxyl groups on adjacent carbon atoms such as carbohydrates, steroids and catecholamines [8]. Boric acid has been used in the form of gel columns for low-pressure separation of catechols [9], and in buffers to stabilise catechols against oxidation. The use of phenyl boronic acid bound to agarose [10] or silica [11] matrices for the high-pressure separation of catechols has also been recently described. This communication demonstrates that addition of alkyl boronates to the mobile phase retards the elution of catechols on a C_{18} reversed-phase column. This permits selective retention of the catecholamines and their catecholic metabolites versus the methoxy-hydroxy metabolites and 5HT and its metabolites on the C_{18} columns already used for most HPLC work.

EXPERIMENTAL

Butane boronic acid (BuB; Sigma or Aldrich) was added to 0.05 or 0.1 *M* sodium phosphate buffers. HPLC was carried out on a Gilson gradient system, using a Rheodyne injector with 200-μl sample loop, and a 15 cm × 4.6 mm column of Hypersil ODS 5 μm with a 5 cm × 2.1 mm guard column, packed with pellicular ODS silica (Chrompack). Fluorescence (Kratos 950; mercury line excitation, interference filter at 254 nm; emission at 365 nm band-pass) and electrochemical (Bioanalytical Systems, LC4 with TL5) detectors were used in series as previously described [12]. Elution was either isocratic, without addition of organic modifier, or a gradient of methanol was run. Buffer A contained no methanol; a linear gradient of 0—100% buffer B (containing 20% methanol) was imposed between 5 and 15 min post-injection and the methanol concentration was returned to zero between 15 and 20 min.

Standards were made up to final dilution in the initial running buffer. Brain tissue was deproteinised in about 10 vols. of 0.1 *M* phosphoric acid and the supernatant neutralised with an equal volume of 0.1 *M* phosphate buffer containing 10 m*M* BuB. This gave a final pH and BuB concentration equal to that of the initial eluting buffer (see below); after a further brief centrifugation, 200 μl of the neutralised extract were injected into the HPLC system.

RESULTS AND DISCUSSION

As would be expected from previous work [9] the effect of BuB was modest at pH 3–6, in the range usually used for HPLC of amine metabolites. The effect became much more marked around neutral pH (7.0–7.5). At this pH, acidic metabolites are less well retained, but by omitting methanol from the running buffer good resolution was achieved. Fig. 1 shows that increasing concentrations of BuB in phosphate buffer, pH 7.5, led to increased retention of catechols, but decreased retention of 5-hydroxyindoles, presumably due to BuB acting as an organic modifier with respect to the latter. 5 m*M* BuB and 0.1 *M* phosphate were selected as conditions to study a larger series of standards (Table I). The retarding effect seems to be greatest for the catecholamines, followed by the catechol metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). The retention of other compounds is reduced, by a factor of about two thirds in most cases. The retention of methylated metabolites [homovanillic acid

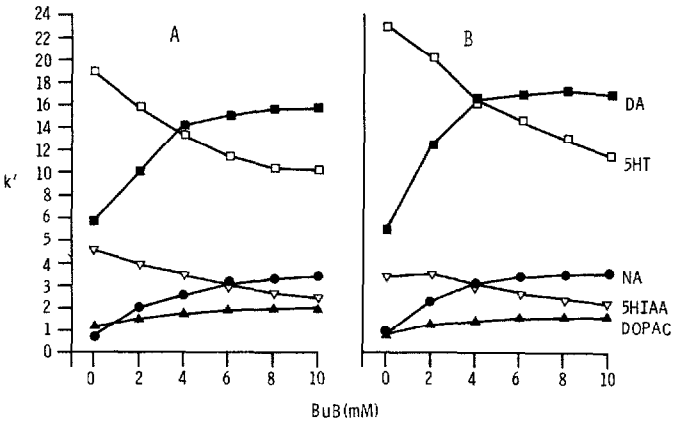

Fig. 1. Effect of increasing concentrations of butane boronic acid on the retention of catechols and 5-hydroxyindoles. Capacity factors (k') are plotted against final BuB concentration in phosphate eluting buffer, pH 7.5, at (A) 0.1 *M* and (B) 0.05 *M*.

TABLE I

EFFECT OF ADDITION OF BUTANE BORONIC ACID (5 m*M*) TO 0.1 *M* PHOSPHATE BUFFER pH 7.5 ON CAPACITY FACTORS FOR CATECHOLS AND OTHER COMPOUNDS

Compound	Capacity factor		Ratio $\frac{\text{with BuB}}{\text{without BuB}}$
	0 m*M* BuB	5 m*M* BuB	
Noradrenaline	0.4	2.15	5.38
Dopamine	3.35	11.8	3.52
3,4-Dihydroxyphenylacetic acid	0.93	1.55	1.68
Vanillylmandelic acid	0.5	0.8	1.60
3-Methoxy-4-hydroxyphenylglycol	7.0	6.18	0.882
Homovanillic acid	5.0	3.6	0.720
Tyrosine	1.55	1.03	0.661
Tryptophan	14.15	9.6	0.678
5-Hydroxyindoleacetic acid	4.2	2.8	0.666
Serotonin	15.0	9.35	0.623
5-Hydroxytryptophol	37.4	22.75	0.608

(HVA), 4-methoxy-4-hydroxyphenylglycol (MHPG)] is reduced less, and vanillylmandelic acid (VMA) is paradoxically retarded as strongly as DOPAC, perhaps due to its α-hydroxy acid structure. Phenyl boronic acid (Sigma) was also tried as a pairing agent, but gave a high electrochemical background.

The method was then applied to the analysis of areas dissected from rat brain. The analysis of NA, DA, 5HT, DOPAC and 5-hydroxyindoleacetic acid (5HIAA) could be carried out in two ways using conditions derived from Fig. 1. At 6 m*M* BuB in 0.05 *M* phosphate with no methanol, isocratic elution, these compounds elute in the order DOPAC, 5HIAA, NA, 5HT, DA. Alternatively, better peak shape for 5HT and DA can be obtained by using 5 m*M* BuB in 0.1 *M* phosphate and running a gradient of 0- 20% methanol, as described under Experimental. Re-equilibration takes 15 min, probably because of disturbance of the equilibrium between the pairing agent and the column.

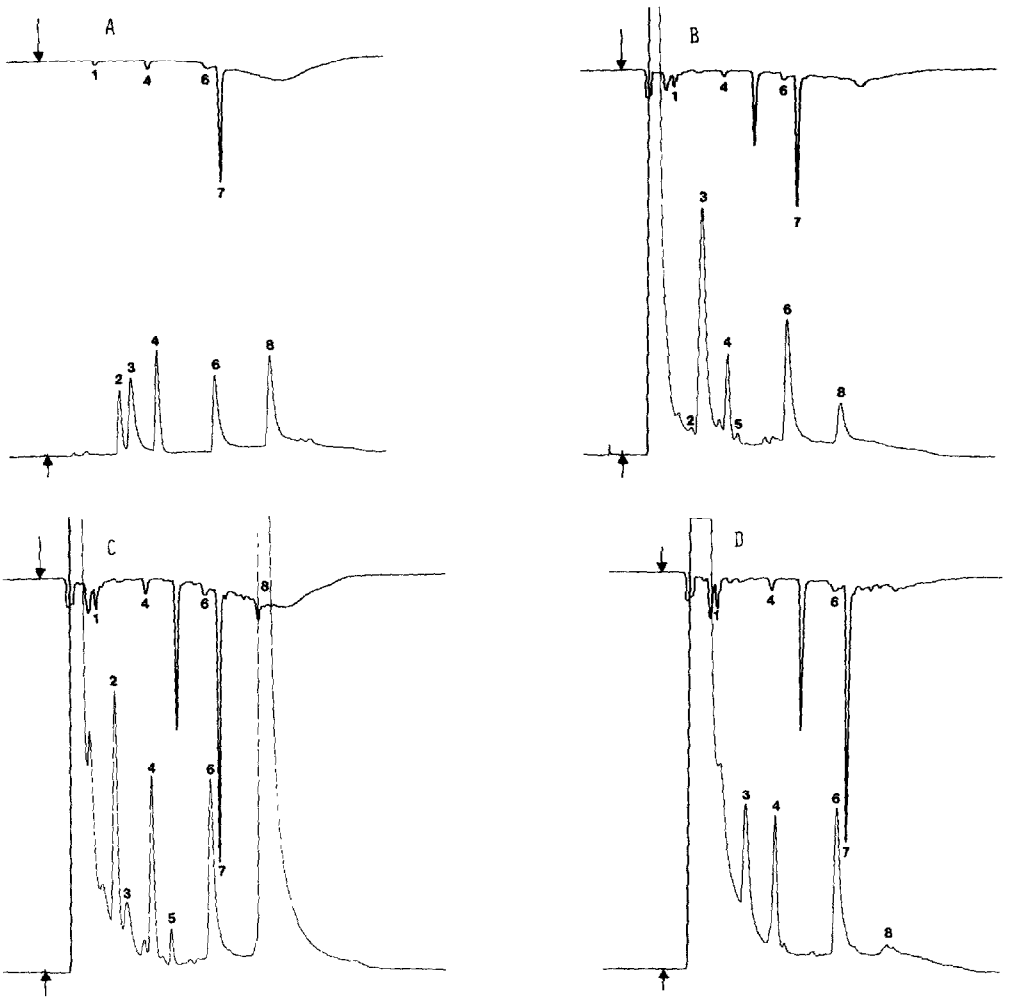

Fig. 2. Separation of amines and related compounds, standards and brain samples. Runnning buffers were 0.1 *M* phosphate, pH 7.5, containing 5 *mM* BuB (final). A gradient of 0—20% methanol was run as described in Experimental. The upper of each pair of traces is obtained by fluorescence detection, sensitivity 0.1, 254/365 nm. The lower is obtained by electrochemical detection in series at 0.4 V, 20 nA full scale. Injection is indicated by arrows. (A) Standards corresponding to 250 ng/g amines and metabolites and 2.5 μg/g amino acids carried through the method. (B) Rat brain hypothalamus. (C) Rat brain striatum. (D) Rat brain hippocampus. Peaks: 1 = tyrosine; 2 = DOPAC; 3 = NA; 4 = 5HIAA; 5 = HVA; 6 = 5HT; 7 = tryptophan; 8 = DA.

This is minimised by keeping the BuB concentration in the second buffer at 5 m*M* final. The elution order is slightly changed, NA eluting between DOPAC and 5HIAA (Fig. 2A). It is important to inject samples in the equivalent of the running buffer (see Experimental), since otherwise a reduction in retention and resolution, especially of NA, is seen, presumably due to the disturbance of the equilibrium between the BuB and the column. The presence of BuB will also help to stabilise catechols against spontaneous oxidation at this relatively high pH.

The working voltage of the electrochemical detector was reduced to 0.4 V

TABLE II

LEVELS OF AMINES, PRECURSORS AND METABOLITES IN RAT BRAIN AREAS

n = 3 in each case.

Compound	Level (μg/g wet weight)		
	Hippocampus	Striatum	Hypothalamus
Tyrosine	13.4	15.6	15.0
Noradrenaline	0.343	0.100	1.528
Dopamine	N.D.*	10.62	0.388
3,4-Dihydroxyphenylacetic acid	N.D.	1.288	N.D.
Tryptophan	5.11	5.67	4.68
Serotonin	0.490	0.478	0.573
5-Hydroxyindoleacetic acid	0.414	0.476	0.463

*N.D. = Not detectable.

so that the unretained peak did not interfere with DOPAC detection. Note that use of a buffer at higher pH results in a downward shift of the oxidation potentials relative to that in the usual pH range [13]. Thus HVA and MHPG, which are also resolved with this system (as expected from Table I), can be detected even at this potential, although sensitivity is greater if a higher working voltage (ca. 0.7 V) is used. Peaks for tyrosine, 5HIAA, 5HT and tryptophan are observed on the fluorescence detector (the amino acids being electrochemically inactive at these working potentials). Fig. 2B—D demonstrates the application of the method to various areas of rat brain. The marked variations, particularly in catecholamine and metabolite levels, between the brain areas analysed (Table II) are in general agreement with those in the literature [1—3, 14].

CONCLUSIONS

Butane boronic acid can be used as a pairing agent in an HPLC mobile phase at around neutral pH to selectively retard the elution of catechol compounds, in particular the catecholamines, from ODS silica. It is likely that the series of alkyl boronic acids will have similar properties. This provides a simple and reversible modification to the properties of these widely used reversed-phase columns, in contrast to the special columns needed if bonded organoboronate phases are used. It thus provides an extra parameter of mobile phase composition which can be manipulated to optimise the separation of complex mixtures of biogenic amines, precursors and metabolites such as are obtained from biological materials. As an example of a group-specific, rather than charge-specific, pairing agent being used in an HPLC mobile phase, BuB may also provide a model for a wide range of applications of other group-specific modifiers in reversed-phase chromatography.

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