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## Note

# Chromatographic separation of catecholic amino acids and catecholamines on immobilised phenylboronic acid

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The chromatographic separation of carbohydrates on an affinity gel containing immobilised phenylboronic acid is a valuable tool in research on nucleotides and saccharides[1,2]. The method is based on complex-formation between the dihydroxyboronyl groups on the gel and the *cis*-diols of the sugar. Catechol compounds also have the molecular configuration for forming such complexes, and this has been extensively used in comparable adsorption on alumina for the purification of catecholamines[3]. Borate-containing eluents have been used in the preparative chromatography of catecholamines on ion-exchange resins, but the eluted boric acid cannot easily be eliminated[4]. Immobilised boronate gel, however, might be useful for separating catechol compounds without contamination by boron.

We have found large amounts of catecholic amino acids, especially cysteinyldopa isomers, in serum and urine of melanoma patients[5,6]. Several major melanin precursors have been purified on alumina with acceptable results, but the recoveries were low and purification of some compounds was incomplete.

By using immobilized phenylboronate gel, we have developed a method permitting good chromatographic separation of catecholic amino acids and catecholamines.

## MATERIALS AND METHODS

### *Chemicals*

Dopa, dopamine, tyrosine, and 3-methoxytyramine were purchased from Sigma (St. Louis, Mo., U.S.A.). Cysteinyldopas were synthesised by the method of Agrup *et al.*[7], and immobilised phenylboronate gel (particle size 0.1–0.4 mm) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). All other chemicals were of the highest purity available, and water triply-distilled from all-glass apparatus was used throughout. All sample solutions were freshly prepared. Sodium phosphate buffers (0.2 *M*) were used for elution at pH 8.0 and 6.8.

### *Procedure*

The gel was allowed to swell in 5% aqueous acetone overnight, and the slurry was packed into a glass column (25 cm × 7 mm I.D.). The eluent was pumped by

an LKB Varioperpex II pump through a Chromatronix loop injector into the column, and elution was monitored by an LKB Uvicord detector operated at 254 nm. Samples of volume 1 ml or less were loaded (by syringe) into a 1-ml loop and then injected on to the column; larger amounts were loaded directly on to the column through the pump. Elution was performed by pumping eluent through the system at 2.5 ml per min. Heterogeneous samples were filtered before loading, and the pH was adjusted with sodium hydroxide. Fractions were collected and analysed fluorimetrically for each catecholic compound; if determination was not performed immediately, sodium metabisulphite was added to prevent oxidation.

## RESULTS AND DISCUSSION

Adsorption of all catecholic compounds on the immobilised phenylboronate gel takes place at neutral or alkaline pH. Since most catechols are easily oxidised at alkaline pH, only a narrow range of pH is available for complex-formation. We found that, for all the catechols investigated, adsorption was complete at pH 8.0 and higher; at slightly lower pH, however, the extent of adsorption varied between the different compounds (see Table I). Thus, dopa was adsorbed quantitatively at pH 8.0, but hardly at all at pH 6.8, whereas 5-S-cysteinyldopa (the major cysteinyldopa isomer in serum and urine from melanoma patients) was completely adsorbed at pH 6.8, but could be quantitatively eluted by triply-distilled water at pH 5.6. Catecholamines such as dopamine were completely adsorbed at pH 6.8 and higher, but, unlike the cysteinyldopas, were not eluted at pH 5.6. Elution of catecholamines was easily achieved with a small volume of dilute mineral acid[8].

TABLE I

ADSORPTION AND ELUTION OF CATECHOLIC COMPOUNDS ON IMMOBILISED PHENYLBORONATE GEL AT DIFFERENT pH VALUES

After application of the test solution, elution was carried out first with the loading buffer, then with water at pH 5.6 and finally with hydrochloric acid (pH 1.75).

| *Substance* | *pH at loading* | *Percentage of substance eluted* | | |
|---|---|---|---|---|
| | | *At pH loading* | *At pH 5.6* | *At pH 1.75* |
| Tyrosine | 8.0 | 100 | 0 | 0 |
| 3-Methoxytyramine | 8.0 | 100 | 0 | 0 |
| Dopa | 8.0 | 0 | 100 | 0 |
| Dopa | 6.8 | 100 | 0 | 0 |
| 5-S-Cysteinyldopa | 8.0 | 0 | 100 | 0 |
| 5-S-Cysteinyldopa | 6.8 | 0 | 100 | 0 |
| Dopamine | 8.0 | 0 | 0 | 100 |
| Dopamine | 6.8 | 0 | 0 | 100 |

The specificity of the column for a catecholic structure is obvious, since neither tyrosine nor 3-methoxytyramine was adsorbed even at high pH. The pH-dependent adsorption of catecholic compounds can be applied in the chromatographic separation of a mixture of catechols by using a pH-step gradient; this is shown in Table II. Fig. 1 shows a chromatogram of a mixture of dopa, 5-S-cysteinyldopa and do-

TABLE II

CHROMATOGRAPHIC SEPARATION OF CATECHOLIC COMPOUNDS ON IMMOBILISED PHENYLBORONATE GEL LOADED AT pH 8.0 AND ELUTED WITH A pH-STEP GRADIENT

| *Substance* | *Percentage of substance eluted* | | |
|---|---|---|---|
| | *At pH 6.8* | *At pH 5.6* | *At pH 1.75* |
| Dopa | 100 | 0 | 0 |
| 5-S-Cysteinyldopa | 0 | 100 | 0 |
| Dopamine | 0 | 0 | 100 |

pamine separated on the phenylboronate gel with the aid of a pH-step gradient. Most other types of chromatographic material used for purification of these easily oxidised and polymerised catechols are destroyed in a single run. We have used the same phenylboronate column almost daily for several months for the purification of both crude urine and synthetic samples without any major loss of capacity.

In applying affinity chromatography on an immobilised phenylboronate gel for the pre-purification of biological fluids, we applied 20 ml of urine (pH 6.8) from a melanoma patient to the column, then washed the column with 140 ml of phosphate buffer solution (pH 6.8), and eluted the cysteinyldopas completely with 160 ml of water at pH 5.6. No cysteinyldopa appeared in the buffer fraction, and only traces in a later acid fraction. Dopa was eluted completely in the buffer fraction, and catecholamines first in the acid fraction. The phenylboronate gel thus gave complete separation of cysteinyldopas from other amino acids and also from dopa and catecholamines. Similar separations are not possible on alumina, and, further, the recovery of 5-S-cysteinyldopa from alumina by elution with 0.1 *M* perchloric acid is *ca.* 35%, whereas recovery from the phenylboronate gel is almost complete.

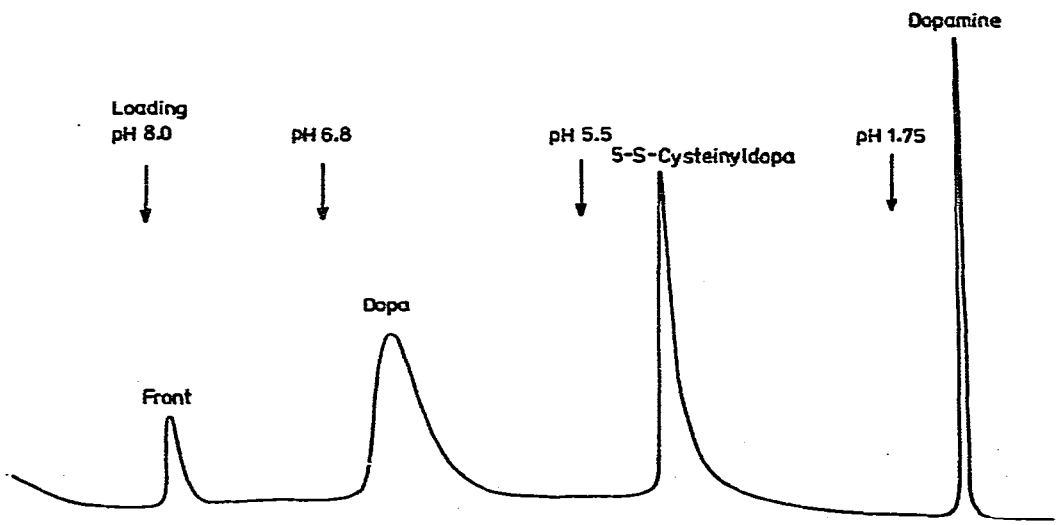


Fig. 1. Chromatogram of a sample containing dopa, 5-S-cysteinyldopa and dopamine separated on an immobilised phenylboronate gel by a pH-step gradient.

Separation of 5-S-cysteinyldopa, dopa and dopamine is possible on an ion-exchange column, but the procedure is time-consuming and the ion-exchange resin does not give a class separation of catecholic from other amino acids and amines in biological fluids. Routine determinations of 5-S-cysteinyldopa, dopa, dopamine and most other catecholic compounds have hitherto involved fluorimetry; interference by fluorophores from different catechols has been a problem, but this can now be eliminated by the separation achieved by the immobilised phenylboronate gel.

The use of HPLC for the separation and subsequent detection of catecholic compounds in biological material needs a pre-purification step, which has hitherto been achieved by adsorption on alumina and elution with acid. This procedure has given good purification of serum[5], but, in analysis for compounds at the subnanogram level in urine, the degree of purification is not generally acceptable. The recovery from alumina is only *ca.* 35% for monocysteinyldopas and less than 10% for, *e.g.*, 2,5-S,S-di-cysteinyldopa with 0.1 *M* perchloric acid as eluent. The use of immobilised phenylboronate gel for pre-purification gives better recoveries and greater purification. Further, both the use of alumina and ion-exchange purification require elution by acid, and acid conditions have been shown to produce artefacts of certain catecholic amino acids; the phenylboronate gel, which gives separation at a pH close to that obtaining in physiological conditions, minimises artefact formation.

In conclusion, the complete adsorption of catecholic compounds at pH 8.0 on immobilised phenylboronate gel, and the separation by a pH-step gradient, provide a simple and effective means for the concentration and purification of dilute solutions. The method described here will undoubtedly be useful in analytical and preparative work with catecholic compounds.

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