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Determination of 3,4-dihydroxyphenylethylene glycol and 3,4-dihydroxyphenylacetic acid in blood plasma by liquid chromatography with amperometric detection*

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Abstract: Liquid chromatography with amperometric detection was employed for the determination of the dihydroxycatecholamine metabolites, 3,4-dihydroxyphenylethylene glycol (DOPEG) and 3,4-dihydroxyphenylacetic acid (DOPAC) in plasma. The compounds were isolated from plasma by adsorption onto alumina, the elution from which was found to be strongly dependent on the acid used. Conditions for the separation on octadecyl-bonded silica were evaluated with particular reference to the influence of pH, ion-pairing anions and quaternary ammonium ions. Recoveries of *ca* 85% and relative standard deviations of about 3% were obtained for the assay of endogenous concentrations.

Keywords: *Liquid chromatography; amperometric detection; dihydroxycatecholamine metabolites; plasma; alumina adsorption.*

Introduction

In previous papers the authors have presented analytical methods for catecholamines in tissue and plasma [1] and in urine [2] based on alumina adsorption and cation-exchange liquid chromatography. However, in many instances the measurement of individual metabolites of the catecholamines may give more relevant information on sympathomimetic activity as, for example, during cardiac infarct or ischemia.

In the present paper methodology has been examined for the determination of the dihydroxycatecholamine metabolites 3,4-dihydroxyphenylethylene glycol (DOPEG) and 3,4-dihydroxyphenylacetic acid (DOPAC) in plasma. Alumina adsorption and liquid chromatography with amperometric detection have been used.

For the assay of DOPEG in plasma a few radioenzymatic methods have been published [3–5], while only one liquid chromatographic method has appeared [6]. For DOPEG in

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brain tissue [7–8] and for DOPAC in plasma [9–10] and tissue [11–14] methods by liquid chromatography and amperometric detection have been recently described.

Experimental

Apparatus

The liquid chromatograph consisted of a Beckman pump Model 112, an injection valve (Rheodyne 7125, Berkeley, CA, USA) with a 60-μl loop and an amperometric detector, BAS model LC 4 or LC 4B (Bioanalytical Systems BAS, West Lafayette, IN, USA). The detector was generally operated at +0.6 V with the Ag/AgCl reference electrode (BAS RE 1) and either a thin-layer cell (BAS TL5A), consisting of a glassy-carbon working electrode, or a dual cell in parallel mode. A Cenco rotary mixer for 56 tubes (Breda, The Netherlands) was used to rotate the tubes during adsorption onto alumina.

Chemicals

3,4-Dihydroxyphenylethylene glycol (DOPEG) and 3,4-dihydroxymandelic acid (DOMA) were obtained from Regis (IL, USA), 3,4-dihydroxyphenyl acetic acid (DOPAC) from Fluka AG (Buchs SG, Switzerland), carbidopa (internal standard) from Merck Sharp & Dohme (Rahway, NJ, USA) and rimiterol (internal standard) from Riker Laboratories (Loughborough, UK). Other reference substances were from Regis or Sigma (St Louis, MO, USA).

Reduced glutathione (GSH) was from Sigma and tris(hydroxymethyl)aminomethane (Tris) analytical grade buffer from Fluka. Alumina, Woelm neutral, was from Woelm Pharma (Eschwege, FRG) and was prepared according to the method given in [1]. *N,N,N*-Trimethyloctylammonium (TMOA) and tetrabutylammonium (TBA) hydrogen sulphate were from the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). All buffer substances and acids were of analytical grade from E. Merck (Darmstadt, FRG).

Analytical procedure

The plasma sample, 2 ml, was transferred to a 4-ml centrifuge tube. 50 μl of ethylenediaminetetraacetate (EDTA; 0.3 mol/l, pH 7), 50 μl of GSH (0.05 mol/l) and 20 mg of alumina were added. While vortexing the tubes 0.20 ml Tris buffer (1 mol/l, pH 8.6) was added and the tubes were then placed in a Cenco rotary mixer and rotated for 15 min. Rimiterol was added to the samples as an internal standard.

The alumina was washed three times by mixing for a few seconds with EDTA solution (3 mmol/l, pH 7). After the final washing the tubes were centrifuged and any excess liquid was again discarded. The compounds were eluted from the alumina with 150 μl of 0.2 mol/l aqueous perchloric acid solution.

After centrifugation the tubes were stored frozen in the dark and thawed just before injection of 50 μl onto the chromatographic column.

Chromatographic system

The separation column was either a 3-μm Supelcosil LC-18-DB (Supelco, Bellefonte, PA, USA) or a home-packed 5-μm Polygosil-C_{18} (Macherey Nagel, Düren, GFR), both 150 × 4.6 mm i.d. Mobile phases consisted of phosphate, acetate or citrate buffer solutions with added ion-pairing agents, as discussed below. Deionized and filtered water (Milli Q, Millipore, Bedford, MA, USA) was used for the mobile phase, which prior to

use was degassed and filtered through a 0.45-μm MF-Millipore filter. The flowrate was 1 ml/min at ambient temperature.

Results and Discussion

Work-up procedure

Batch extraction of the samples with alumina was chosen as the work-up procedure in order to isolate the dihydroxycatecholamine metabolites from *o*-methylated compounds and other electroactive substances in the samples. In a previous paper [1] a recovery of about 85% was reported for the alumina adsorption of the catecholamines. In the present studies, however, different recoveries were obtained by elution from alumina, using different acidic aqueous solutions. With perchloric acid low recoveries were obtained for DOMA, while the recovery for DOPEG and DOPAC was satisfactory (cf. [10, 15]). By increasing the perchloric acid concentration from 0.2 to 0.4 mol/l an increase in the recovery of DOMA was achieved, but by changing to phosphoric or sulphuric acid solution a much more pronounced effect was observed (Fig. 1). However, these two acids led to a broader 'solvent-front' peak, thus counteracting the possibility to estimate the DOPEG peak in the chromatogram. Since it has not been found possible to overcome this problem, perchloric acid is employed for the assay of DOPEG and DOPAC, for which the recoveries are in the range 81–88%, as compared to a directly injected reference sample.

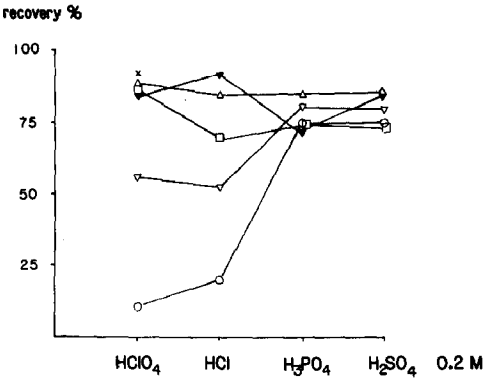

Figure 1
Influence of different acidic eluents on the recovery from alumina. Key: ○ DOMA; △ DOPEG; □ DOPAC; ▽ 3,4-dihydroxybenzoic acid; ▼ carbidopa; × rimiterol.

Chromatographic system

The norepinephrine metabolites in reference solutions could be separated by the addition to the mobile phase of perchlorate or sulphonates, which increase the retention of amino compounds. The effect of butanesulphonate is illustrated in Fig. 2. These separations were performed with a Polygosil-C_{18} column and intended for use in the assay of perfusate solutions.

As regards plasma samples, DOPEG was not sufficiently retained on a number of other columns tested. The selectivity pattern and the performance of compounds with intact amino function had to be considered. In this study most work was carried out with Supelcosil LC-18-DB and Polygosil-C_{18} columns which were found to largely fulfil the requirements both for DOPEG and DOPAC.

It can be anticipated that the retention of DOPEG should not be affected to any great extent either by pH changes within the range of 2–7, or by the addition of ion-pairing agents to the mobile phase. However, these methods for regulating the retention of ionic

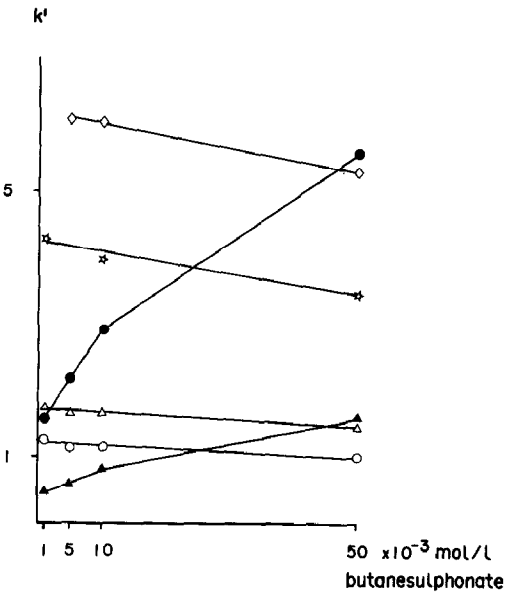

Figure 2
Influence of butanesulphonate on retention. Stationary phase: 5-μm Polygosil-C_{18}, in 150 × 4.6 mm i.d. column; mobile phase: butanesulphonate in phosphate buffer, pH 2.4 (including 0.01% m/v EDTA). Key: ○ DOMA; △ DOPEG; ▲ norepinephrine; ● normetanephrine; ⋆ 4-hydroxy-3-methoxy mandelic acid; ◇ 4-hydroxy-3-methoxyphenylethylene glycol.

species, particularly the catechols, can be exploited so that interference with DOPEG in the chromatogram is avoided. As expected pH variation influences the retention of the acids, as shown in Fig. 3. Although less than 1% is adsorbed onto alumina, uric acid is of major concern, since it is present in relatively large amounts. By pH regulation alone it was difficult to find conditions for isolating the DOPEG peak in plasma samples, without interference from other components.

As expected, addition of a quaternary ammonium ion, TBA or TMOA, to the mobile phase increases the retention of anionic species in the sample, such as the anionic forms of DOPAC and DOMA, for example. As can be seen in Fig. 4 the effect of TBA is most pronounced at low concentrations, while the retention of uric acid is almost unaffected. A beneficial effect of the quaternary ammonium ions is that the amino compounds are displaced from the bonded phase and eluted early in the chromatogram (cf. Fig. 4). By the addition of TBA to the mobile phase (pH 5.7), it was possible to separate the peaks for DOPEG and DOPAC in the plasma samples tested, as shown in Fig. 5 (human

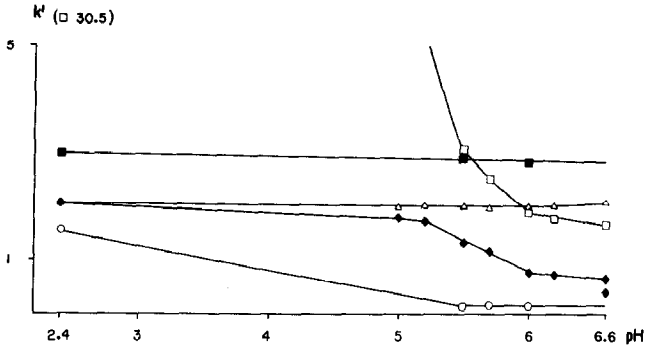

Figure 3
Influence of mobile phase pH on retention. Stationary phase: 3-μm Supelcosil LC-18-DB; mobile phase: citrate or phosphate buffer solution. Key: ○ DOMA; △ DOPEG; □ DOPAC; ■ dopamine; ◆ uric acid.

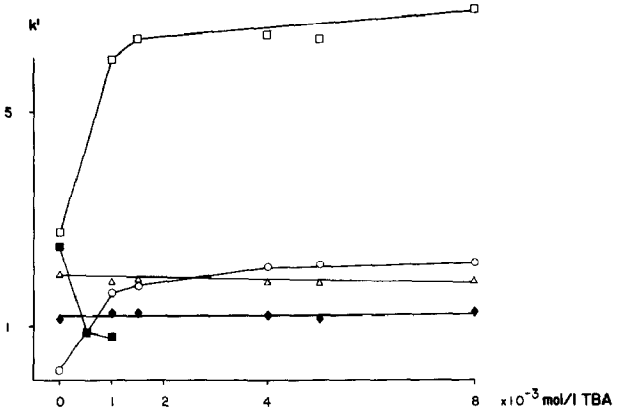

Figure 4
Influence of tetrabutylammonium (TBA) on retention. Stationary phase: 3-μm Supelcosil LC-18-DB; mobile phase: TBA in citrate buffer, pH 5.7. Key: ○ DOMA; △ DOPEG; □ DOPAC; ■ dopamine; ◆ uric acid.

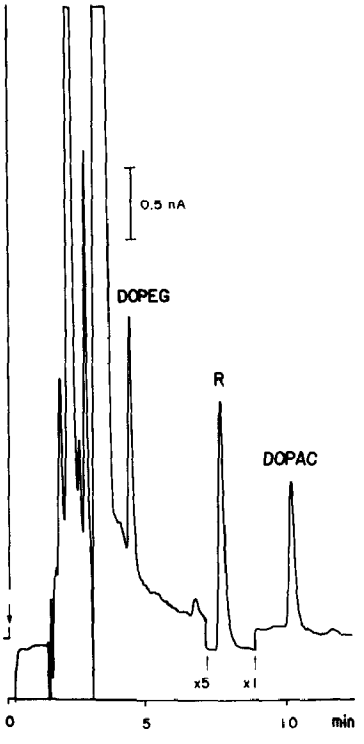

Figure 5
Chromatogram of 2 ml human plasma containing 8.45 pmol/ml DOPEG and 9.26 pmol/ml DOPAC. Stationary phase: 3-μm Supelcosil LC-18-DB; mobile phase: $5 \cdot 10^{-4}$ mol/l tetrabutylammonium in citrate buffer, pH 5.7. Amperometric detector potential: +0.6 V.

plasma) and in Fig. 6 (dog plasma). In this system DOMA was eluting before DOPEG and too close to the solvent front, so that in the present method the simultaneous determination of DOPEG and DOMA is not possible.

With TBA in the mobile phase, it is not only pH that is of importance for the retention of the acids, but also the buffering ions play a significant rôle. This is shown in Fig. 7, where capacity factors (k') for a number of compounds are given for acetate, citrate (pH 5.7) and phosphate (pH 6.0) as buffering anions. The differences can probably be attributed to the competition, exerted by the buffer anions as ion pairs with TBA, for the adsorption sites on the bonded phase.

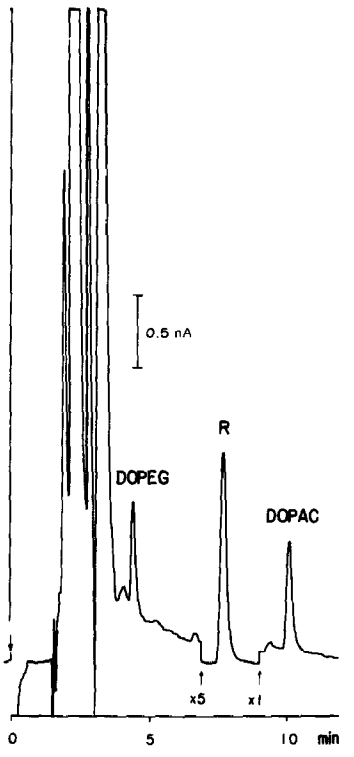

Figure 6
Chromatogram of 2 ml dog plasma containing 3.72 pmol/ml DOPEG and 6.95 pmol/ml DOPAC. Chromatographic conditions as in Fig. 5.

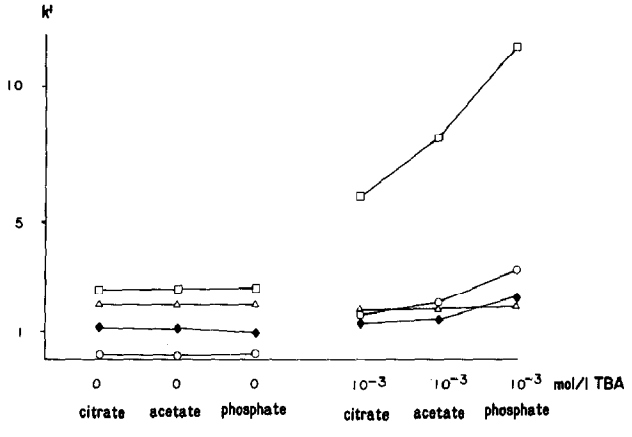

Figure 7
Influence of the buffer anion on retention. Stationary phase: 3-μm Supelcosil LC-18-DB; mobile phase: tetrabutylammonium (TBA) in buffer solution pH 5.7 (citrate, acetate) or pH 6.0 (phosphate). ○ DOMA; △ DOPEG; □ DOPAC; ◆ uric acid.

Detection

The chromatographic systems that performed satisfactorily for reference solutions were tested on plasma samples from different individuals. In some cases it was difficult to establish whether or not the peaks were disturbed by interfering sample components, a point illustrated in the chromatogram of a human plasma sample in Fig. 8. Separation on different phase systems may then give further proof of interference or peak purity. An

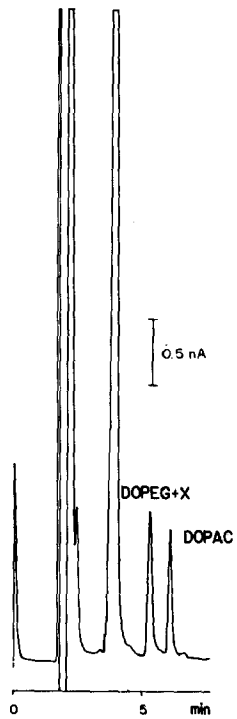

Figure 8
Chromatogram of a human plasma sample where an interfering compound co-elutes with DOPEG. Stationary phase: 3-μm Supelcosil LC-18-DB; mobile phase: citrate buffer, pH 5.7. Amperometric detector potential: +0.5 V.

additional method where dual-cell operation is used in parallel mode was employed for the sample shown in Fig. 8. The quotients for the response at 0.3 and 0.5 V for DOPEG were in this case much higher in human and dog plasma than in the reference solution, indicating the presence of an additional component under the DOPEG peak.

It is interesting to note that the detector response was influenced by the perchloric acid content in the injection solution itself, as shown in Fig. 9. Although DOPEG was relatively unaffected, the detector response to the amines increased and that to the acids decreased, after considering minor changes in the retention. Injected perchlorate is probably retained as an ion pair with TBA, which affects the electrochemical reaction in the detector cell; the effects on peak symmetry or plate height seem to be negligible.

Ruggedness and precision

The presence of tetrabutylammonium (TBA) in the mobile phase decreased the long-term stability of the chromatographic columns. By repacking the top of the column the performance could be restored until such time as the column had to be replaced. A guard column should contribute to improved stability.

The internal standards, 3,4 dihydroxybenzoic acid, rimiterol and carbidopa, were not ideal because of the variation in detector response with the acid strength in the injection solution (Fig. 9). Moreover, rimiterol was found to be susceptible to any loss of chromatographic performance of the column, as illustrated in Fig. 10, which shows a chromatogram of a reference solution.

The relative standard deviations for assays at endogenous levels (about 9 nmol/l) of DOPEG and DOPAC in identical human plasma samples were 3.0 and 3.9% respectively (n = 8). The linear regression data for standard curves of DOPEG and

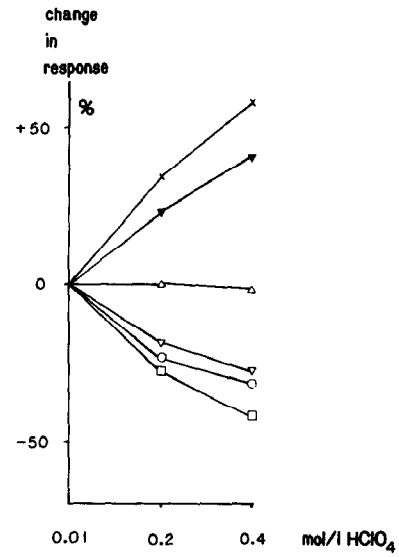

Figure 9
Influence of the concentration of perchloric acid in the injection solution on detector response. Chromatographic conditions were as in Fig. 5. Key: ○ DOMA; △ DOPEG; □ DOPAC; ▽ 3,4-dihydroxybenzoic acid; ▼ carbidopa; × rimiterol.

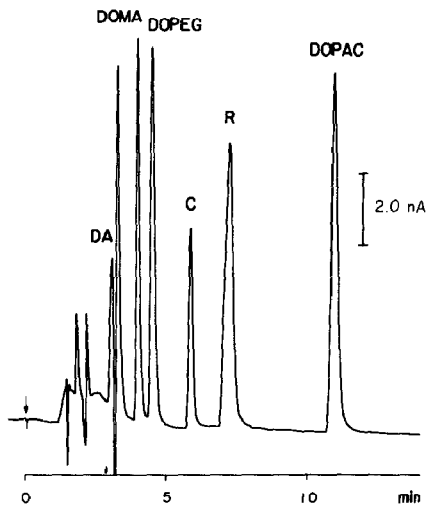

Figure 10
Chromatogram of a reference sample containing dopamine (DA), DOMA, DOPEG, DOPAC and carbidopa (C) and rimiterol (R) tested as internal standards. Chromatographic conditions as in Fig. 5.

DOPAC were, respectively: DOPEG: $y = 0.237x - 1.54$; DOPAC: $y = 0.185x - 1.53$. (x: 1–1000 nmol/l; y: nA; $n = 10$).

The recovery from the alumina adsorption process was reproducible and found to be the same both for plasma and for pure aqueous samples. An internal standard added to the perchloric acid extractant could thus be used to compensate for any minor variation in elution volume and detector response. 3-Methoxy-4-hydroxyphenylethylene glycol, which is present in plasma, is not adsorbed onto alumina and is suitable as an internal standard from the point of view of retention and detection.

Thus a satisfactory LC method for the simultaneous determination of dihydroxycatecholamines in plasma has been developed. The method is characterized by relative simplicity, ruggedness and high sensitivity for monitoring catecholamine metabolites in studies on the disposition of sympathomimetic amines in man.

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