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LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF 3,4-DIHYDROXYPHENYLETHYLENE GLYCOL AND 3,4-DIHYDROXYMANDELIC ACID IN PLASMA

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SUMMARY

A method is described for the simultaneous determination of the deaminated catecholamine metabolites 3,4-dihydroxyphenylethylene glycol (DOPEG) and 3,4-dihydroxymandelic acid (DOMA) in plasma by liquid chromatography with amperometric detection. The compounds are extracted from plasma by adsorption on alumina, then separated on a reversed-phase column coated with tributyl phosphate as the stationary liquid phase. It is a simple and selective method that permits the determination of basal levels of DOPEG and DOMA in plasma with a relative standard deviation of 3%.

INTRODUCTION

Measurement of the deaminated metabolites of norepinephrine may lead to a better understanding of the activity of the sympathetic nervous system. In contrast to the large number of different techniques available for determination of catecholamines, only a few methods have been developed for measuring the metabolites 3,4-dihydroxyphenylethylene glycol (DOPEG) and 3,4-dihydroxymandelic acid (DOMA) in plasma. Some radioenzymatic methods allow the determination of DOPEG[1,2] or DOPEG and DOMA simultaneously[3,4] but no liquid chromatographic method for the determination of DOMA in plasma has been described and just a few are available for DOPEG[5–7]. Possible reasons for this are difficulties in obtaining acceptable recoveries, especially for DOMA[6,8], and problems in separating DOPEG from early eluting compounds in the chromatograms.

EXPERIMENTAL

Apparatus

The liquid chromatograph was composed of a Constametric II G LC pump from LDC (Riviera Beach, FL, U.S.A.) with extra pulse dampers, an ISS-100 automatic injector from Perkin-Elmer (Überlingen, F.R.G.), with a refrigerated sample tray, a model 4270 integrator from Spectra-Physics (San Jose, CA, U.S.A.) and an M 460 electrochemical detector from Waters Assoc. (Milford, MA, U.S.A.), which

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was combined with a TL-5A thin-layer cell, from Bioanalytical Systems (BAS) (West Lafayette, IN, U.S.A.), composed of a glassy carbon working electrode, a stainless-steel auxiliary electrode and a silver–silver chloride reference electrode. A Cenco (Breda, The Netherlands) rotary mixer was used for the alumina adsorption and a Model 2601 multi-tube vortexer (SMI Emeryville, CA, U.S.A.) for the elution step.

Chemicals

DOPEG, DOMA, 3,4-dihydroxyphenylethanol (DOPET) and 3-methoxy-4-hydroxyphenylethanol (MOPET) were obtained from Regis (Morton Grove, IL, U.S.A.). Dopamine (DA) hydrochloride, reduced glutathione (GSH), sodium thioglycolate and ethylene glycol bis (β-aminoethyl ether)-N',N'-tetraacetic acid (EGTA) were purchased from Sigma (St Louis, MO, U.S.A.); 3-methoxy-4-hydroxybenzyl alcohol (MHBOH) was obtained from EGA-Chemie (Steinheim, F.R.G.), isovanillinemandelic acid (iso-VMA) from Aldrich (Milwaukee, WI, U.S.A.) and tris(hydroxymethyl)aminomethane (Tris, analytical-reagent grade), boric acid, 3,4-dihydroxyphenylacetic acid (DOPAC) and methanesulphonic acid from Fluka (Buchs, Switzerland). Alumina (Woelm neutral) was purchased from Woelm Pharma (Eschwege, F.R.G.) and was purified and activated according to the method given in ref. 9. Tetrabutylammonium (TBA) hydrogen sulphate was obtained from the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden), pentanesulphonic acid (HPLC grade) from Fisons (Loughborough, U.K.), disodium ethylenediaminetetraacetate (EDTA), sodium hydrogen sulphite, ascorbic acid, tartaric acid, citric acid, tri-*n*-butyl phosphate (TBP) and all buffer substances and inorganic acids were of analytical-reagent grade from E. Merck (Darmstadt, F.R.G.).

Sample preparation and work-up procedure

Blood was collected in evacuated 5-ml tubes containing 100 μl of a solution of 0.2 *M* GSH and 0.2 *M* EGTA. The samples were centrifuged at 4°C for 5 min at 1000 *g* and the plasma phases were separated and stored at −70°C.

A 20-mg amount of alumina and 50 μl of each of the stabilizing agents, 0.3 *M* EDTA and 0.05 *M* GSH, were added to 2 ml of plasma. While mixing, the pH was adjusted to 8.6 by addition of 0.2 ml of 1 *M* Tris buffer (pH 8.6), then the contents of the tubes were mixed by rotation (40 turns/min) for 30 min. After washing the alumina twice for a few seconds with 2 ml of 0.003 *M* EDTA solution, DOPEG and DOMA were eluted with 150 μl of phosphate buffer (pH 2.2, $I = 0.2$) containing GSH (5 mmol/l) and MOPET (0.5 μmol/l) as an internal standard. A 50-μl aliquot was injected onto the chromatographic column.

Chromatography

The chromatographic separation was performed on a 5-μm Nucleosil C_{18} column (150 × 4.6 mm I.D.) (Macherey-Nagel, Düren, F.R.G.). The column had been coated with a TBP liquid stationary phase by injecting 500 μl of TBP at a rate of *ca.* 5 μl/min. The mobile phase, which was a citrate buffer (pH 3, $I = 0.1$), was saturated to 90% with TBP by diluting a saturated solution. By recirculating the mobile phase overnight, the chromatographic system was equilibrated and constant retention times were obtained. The flow-rate was 1 ml/min and the eluent was monitored with an electrochemical detector operated at 0.7 V. Both the column and the detector cell

were kept in a Faraday cage, which was thermostated to keep the temperature constant to within ±0.1°C throughout the day. A temperature of 23°C was chosen.

A 3-μm Supelcosil C_{18} column (150 × 4.6 mm I.D.) from Supelco (Bellefonte, PA, U.S.A.) was used to verify the selectivity of the separation system. The mobile phases for this column were either citrate buffer (pH 5.7) with a TBA concentration of $5 \cdot 10^{-3}$ mol/l or citrate buffer (pH 6) containing 10^{-3} mol/l of TBA.

RESULTS AND DISCUSSION

Work-up procedure

The work-up procedure is similar to that described for catecholamines[9]. However, in order to obtain acceptable recoveries of DOPEG and especially of DOMA in the alumina extraction, the choice of eluent was of great importance. As reported earlier[6], perchloric acid, which was previously used for the elution of catecholamines, gave a low recovery of DOMA. A low recovery has also been noted when hydrochloric acid was used as an eluent[8], but this was interpreted as being caused by decomposition. Our studies indicate that the low recovery was due to incomplete desorption from the alumina.

For a first indication of the desorbing capacity of different eluents, 20 mg of alumina were mixed with 150 μl of solutions containing DOPEG and DOMA, then the amounts not adsorbed on the alumina were measured. Table I shows the fractions of DOPEG and DOMA not adsorbed as a percentage of the initial concentrations. The desorption of DOMA was particularly influenced to a great extent by the nature of the eluent. Perchloric acid, hydrochloric acid, sulphuric acid, boric acid and the sulphonic acids induced adsorption of DOMA on alumina and thus gave low recoveries. Of the remaining eluents, phosphoric acid and the phosphate buffer solution were the two alternatives to consider, as tartaric acid showed an interfering peak in the chromatogram after the work-up procedure, and citric acid lowered the detector response of DOPEG. Recoveries of the work-up procedure for DOPEG and DOMA

TABLE I

INFLUENCE OF THE ELUENT ON THE ADSORPTION OF DOPEG AND DOMA ON ALUMINA

Eluent	*Concentration (mol/l)*	*Fraction not adsorbed (%)*	
		DOPEG	*DOMA*
Perchloric acid	0.2	95	11
Hydrochloric acid	0.2	72	18
Phosphoric acid	0.2	70	65
Sulphuric acid	0.2	40	42
Citric acid	0.5	76	71
Boric acid	0.5	63	1
Tartaric acid	0.5	100	77
Methanesulphonic acid	0.2	83	12
Pentanesulphonic acid	0.1	43	2
Phosphate buffer (pH 3, I = 0.2)		99	82

with 0.2 and 0.5 *M* phosphoric acid and phosphate and citrate buffers (pH 2–4, $I = 0.1$–0.5) were determined. The final choice of eluent was a phosphate buffer (pH 2.2) with an ionic strength of 0.2, as it showed both good recovery and stability for DOPEG and DOMA. The pH of the eluent after the desorption procedure was about 3.

Other factors studied were the rotation time for the adsorption process, the concentrations of the stabilizing agents, EDTA and GSH, the amount of alumina, the volume of eluent and the number of washings. Rotation times from 3 to 45 min were tested; the results showed that rotation for more than 30 min did not affect the recovery. Also it was not improved by increasing the concentrations of EDTA and GSH or by doubling the amount of alumina or the volume of eluent. The number of washings did not seem to have much effect on the chromatographic results, and in the method described two washings were used. For the concentration range tested (1–1000 pmol/sample) linearity was found for both DOPEG and DOMA in aqueous and plasma samples.

Stability

Precautions had to be taken to avoid the decomposition of DOPEG and DOMA in reference solutions and in injection vials. DOMA, in particular, is labile and the choice of temperature, injection vials and eluents were of great importance. Reference solutions of DOPEG and DOMA in phosphoric acid and in phosphate buffers (pH 2–6) were stable overnight at room temperature. For longer storage, the greatest stability was observed in a buffer of pH 3. Addition of sodium hydrogen sulphite did not improve the stability, and in both phosphate buffer (pH 6) and 0.2 *M* phosphoric acid it was even found to be disadvantageous.

In order to avoid decomposition in the injection vials, the automatic injector was equipped with a refrigerated sample tray. Antioxidants, such as ascorbic acid, thioglycolic acid, and sodium hydrogen sulphite were abandoned, as they gave large front peaks in the chromatograms. EDTA could not be used, as it lowered the response of DOMA in phosphate buffer (pH 2). However, GSH was efficient without interfering with detection.

When small, conical, plastic vials were used, the response was inaccurate and irreproducible after storage at −20 or −70°C. This was due to the concentration gradient formed during freezing and difficulties in mixing the thawed solutions in the conical vials. In small glass vials the response of low concentrations of DOPEG and DOMA in 0.01 *M* phosphoric acid was lowered after storage at −20°C. However, the concentration increased if the injection vials were allowed to stand for some time before injection, indicating that the decline in response was due to adsorption on the glass surface. This adsorption was avoided by using a buffer solution instead of pure acid. We preferred to use injection vials of borosilicate glass (0.3 ml) (Chromacol, London, U.K.) and, to increase the stability, we added 5 mmol/l of GSH to the eluent. The samples were stable for two days in the refrigerated injector. If the worked-up samples had to be stored before injection they were kept at −70°C.

Chromatography

DOPEG is difficult to separate chromatographically from other plasma components in a crude extract, as its retention cannot be affected by either pH changes

or ion-pairing agents in the mobile phase. One way to obtain an adequate separation from the front peak is to add tetrabutylammonium to the mobile phase, which will displace and squeeze the amino compounds together in the front[6]. However, with that method DOMA could not be determined simultaneously with DOPEG. In the present work, we therefore utilized the strong hydrogen-accepting agent TBP as a liquid stationary phase, which provides columns of good stability. It has the advantage of retaining hydrogen-donating compounds such as carboxylic acids whereas, in the absence of suitable counter ions, the amines are not retarded[10–12]. The changes in the retention with the mobile phase pH are shown in Fig. 1. In this initial study, a Polygosil C_{18} column was used, the properties of which are similar to those of Nucleosil C_{18}.

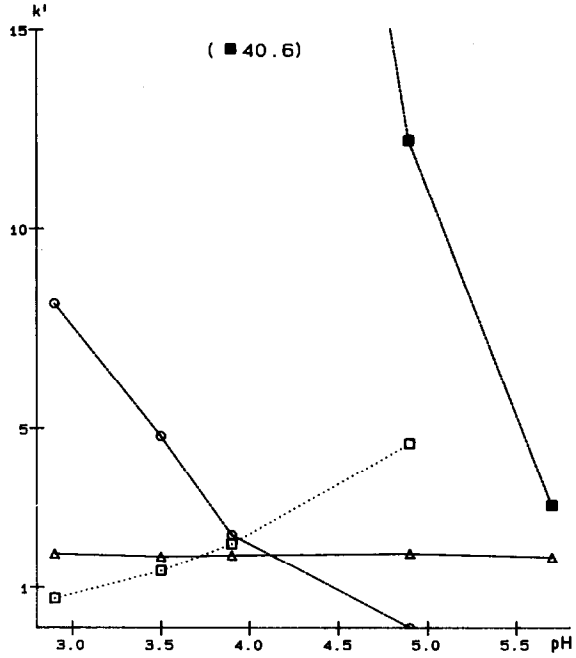

Fig. 1. Influence of pH of the mobile phase on retention. Stationary phase, Polygosil C_{18} (5 μm) coated with TBP; mobile phase, citrate buffer (I = 0.1), 90% saturated with TBP; potential, 0.7 V. ○, DOMA; △, DOPEG; □, DA; ■, DOPAC.

Figs. 2 and 3 show that clean chromatograms were obtained from a reference sample and a human plasma sample, respectively. DOPAC, with a retention time of *ca.* 1 h, had to be taken into consideration, because it could interfere with a subsequent chromatogram. In all the plasma samples analysed an unknown peak appeared, the retention of which was influenced by the mobile phase pH in a similar way to that of DOMA (Fig. 4). At a pH of 3 the compounds could be separated, as can be seen in Fig. 3. The response of the unknown compound was much higher than that of DOMA, but it was decreased significantly by the addition of GSH to the tubes used for sample collection and to the eluent used for desorption from alumina.

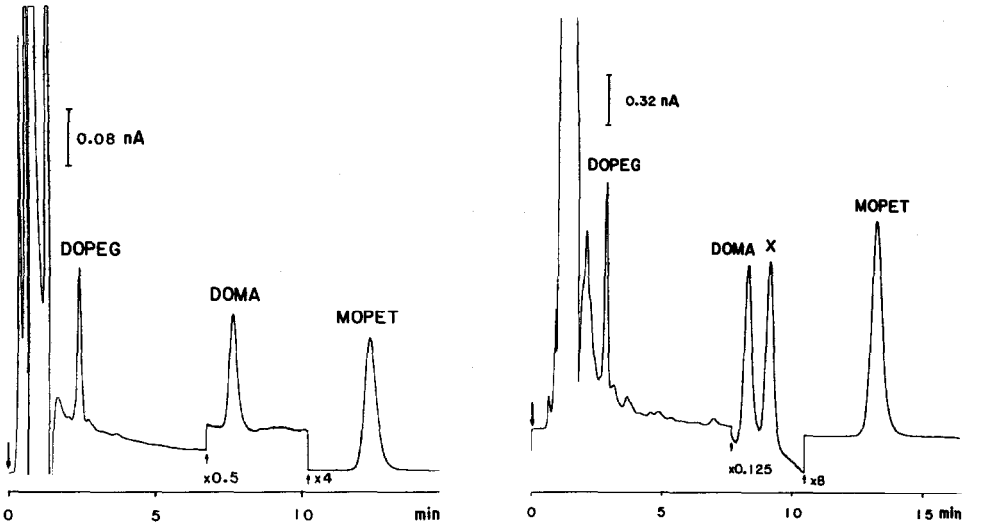

Fig. 2. Chromatogram of a reference sample containing 2.47 pmol of DOPEG, 2.08 pmol of DOMA and 20.6 pmol of MOPET. Stationary phase, Nucleosil C_{18} (5 μm) coated with TBP; mobile phase, citrate buffer (pH 3.0, I = 0.1), 90% saturated with TBP; potential, 0.7 V.

Fig. 3. Chromatogram of a human plasma sample containing 8.69 pmol/ml of DOPEG and 1.97 pmol/ml of DOMA. Chromatographic conditions as in Fig. 2.

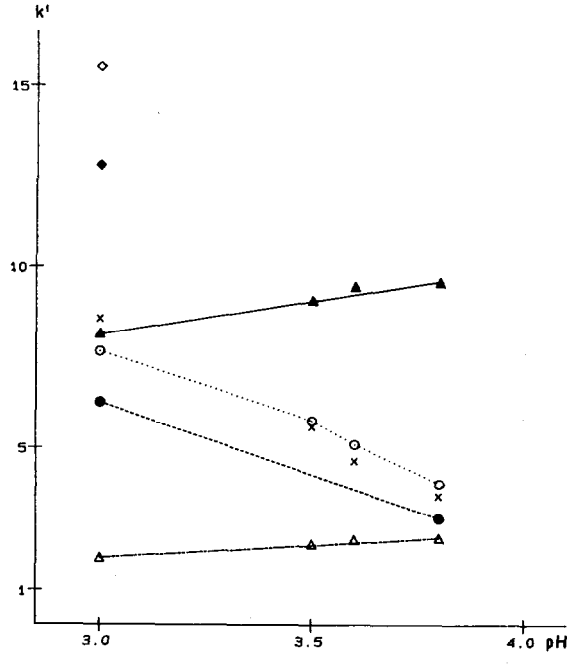

Fig. 4. Influence of pH of the mobile phase on retention. Stationary phase, Nucleosil C_{18} (5 μm) coated with TBP; mobile phase, citrate buffer (I = 0.1), 90% saturated with TBP; potential, 0.7 V. ○, DOMA; △, DOPEG; ◇, DOPET; ●, iso-VMA; ▲, MHBOH; ◆, MOPET; ×, unknown.

Response

It is well known that amperometric detection is influenced by the ambient temperature. Fig. 5 shows the changes in response with temperature over a period of time. This experiment was performed without a temperature-controlled injector. DOMA was probably partially decomposed, because its response, in contrast to that of DOPEG, did not revert to the initial value. The magnitude of the variation of the response in our system was about 4%/°C for DOPEG and 8% for DOMA. Because of the large temperature differences in the laboratory, we thermostated the chromatographic column and the cell components. A cupboard insulated with lagging material was thermostated. A blower circulating the electrically heated air and cold water circulating through copper tubes maintained a constant temperature of 23 ± 0.1°C.

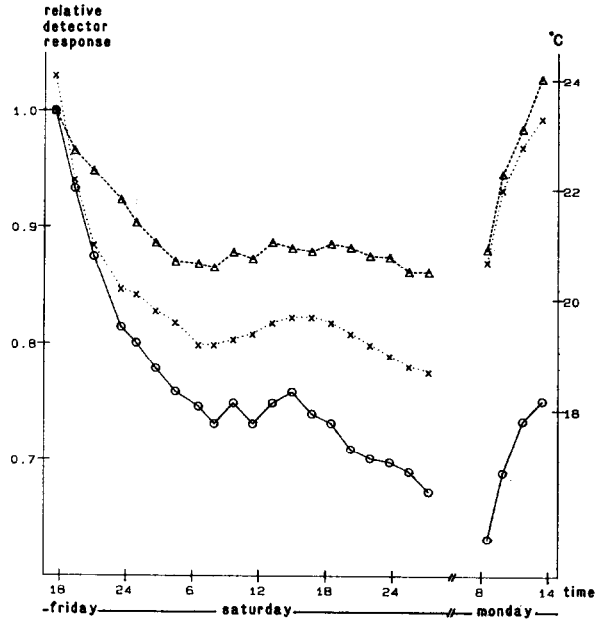

Fig. 5. Change of response with temperature over a period of time. ○, DOMA; △, DOPEG; ×, temperature (°C).

An internal standard was added to compensate for variations in the elution and injection volumes. Iso-VMA, MHBOH, MOPET and DOPET were tested. DOPET was assumed to compensate for the whole work-up procedure, as it is a dihydroxyphenyl compound and will be adsorbed on alumina. However, the recovery of DOPET from plasma samples was lower than that of DOPEG and DOMA and, further, it was eluted late in the chromatogram, as shown in Fig. 4. Of the methylated compounds that were added to the eluent, MHBOH interfered with DOMA in the chromatogram (Fig. 4) and iso-VMA was adsorbed to some extent on alumina. Among the substances tested, we found MOPET to be best suited as an internal standard.

Recovery, precision and accuracy

Reference samples containing two different concentrations of DOPEG and DOMA and ten identical human plasma samples were analysed according to the procedure and the results were compared with the responses of the corresponding compounds in a reference solution that was injected directly into the chromatographic column. The results for the absolute recovery and precision are given in Table II. The recoveries were 70–80% and the relative standard deviations for basal levels of DOPEG and DOMA in plasma were 1–2% and 2–3%, respectively. Limits of determination for DOPEG and DOMA were 0.5 pmol/ml. Because the recovery from plasma samples was lower than that for reference samples, standard additions were used in the determination of unknown plasma concentrations.

TABLE II

ABSOLUTE RECOVERY AND RELATIVE STANDARD DEVIATION (R.S.D.) FOR DOPEG AND DOMA IN REFERENCE SOLUTIONS AND PLASMA SAMPLES

The plasma sample contained 8.69 pmol/ml of DOPEG and 1.97 pmol/ml of DOMA.

Substance	*2 pmol/sample (n = 10)*		*20 pmol/sample (n = 8)*		*Plasma (n = 10)*	
	Recovery	*R.S.D. (%)*	*Recovery*	*R.S.D. (%)*	*Recovery*	*R.S.D. (%)*
DOPEG	80	2.9	78	2.3	75	2.6
DOPEG/MOPET		2.8		2.2		1.4
DOMA	75	3.2	76	3.3	67	2.9
DOMA/MOPET		1.9		2.2		2.5
MOPET	88	2.0	88	2.9	92	3.0

We found plasma levels of about 8 pmol/ml of DOPEG and 2 pmol/ml of DOMA in the samples assayed. The concentration of DOMA was 5–10 times lower than values reported earlier[3,4], whereas the concentrations of DOPEG were in good agreement. In order to verify our data, plasma samples were also analysed by two other chromatographic systems, composed of a 3-μm Supelcosil C_{18} column with either a citrate buffer (pH 5.7), containing $5 \cdot 10^{-3}$ mol/l of TBA as mobile phase, or a citrate buffer (pH 6) with a lower TBA concentration of 10^{-3} mol/l. With these systems, DOPEG could not be determined but the concentration of DOMA proved to be the same as with our TBP system, supporting the accuracy of this result. The values of DOMA in refs. 3 and 4, which seem to be too high, were determined by radioenzymatic methods using thin-layer chromatography as the separation system. The R_F values reported for DOMA in the separation steps were very low. This means that there was hardly any separation from the starting zone and, consequently, DOMA may have been contaminated.

CONCLUSION

As far as we know, the method described here is the first liquid chromatographic method for determining DOMA in plasma. In addition, DOPEG can also

be determined. The use of TBP as a stationary phase allows the separation of these polar compounds from other components in an alumina extract of plasma. The low recovery of DOMA reported earlier was avoided by using a buffer solution instead of an acid as the eluent.

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