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Method for the determination of γ-L-glutamyl-L-dihydroxyphenylalanine and its major metabolites L-dihydroxyphenylalanine, dopamine and 3,4-dihydroxyphenylacetic acid by high-performance liquid chromatography with electrochemical detection

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SUMMARY

A high-performance liquid chromatographic method for the analysis of γ-L-glutamyl-L-dihydroxyphenylalanine (gludopa) and its major metabolites L-dihydroxyphenylalanine (L-DOPA), dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) is described. High sensitivity is achieved with a multi-cell coulometric detector utilising the specific electrochemical properties of gludopa (limit of detection 10 pg on-column). The retention time of gludopa was both pH-dependent and sensitive to negatively charged ion-pairing agents. An alumina-based solid-phase sample preparation technique with dihydroxybenzylamine as internal standard is described for plasma and urine (limit of detection 40 pg/ml) and an ultrafiltration technique is described for tissues (limit of detection 1–10 ng/g). After treatment with 50 mg/kg gludopa, in excess of twenty separate catecholic metabolic peaks can be detected in rat urine, whereas in humans after 9 mg/kg the only catechols detected were L-DOPA, dopamine and DOPAC.

INTRODUCTION

The dipeptide γ-L-glutamyl-L-dihydroxyphenylalanine (gludopa, Fig. 1) is an example of a prodrug which is believed to be specific to the kidney [1]. Dopamine (DA) is the active agent liberated by the sequential enzymatic ac-

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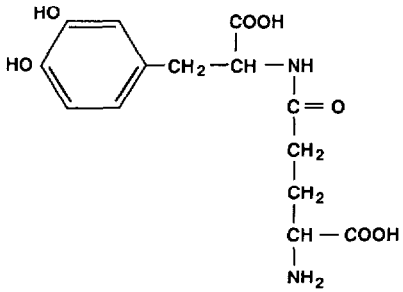

Fig. 1. Structure of γ-L-glutamyl-L-dihydroxyphenylalanine (gludopa).

tion of γ-glutamyltranspeptidase (γ-GT, EC 2.3.2.2) and L-DOPA decarboxylase (EC 4.1.1.26). The basis for kidney specificity comes from indirect measurements of tissue enzyme activities, tissue uptake of [^{14}C]glutamyl amino acids [2–4] and from urinary excretion of DA [5]. To date, no analytical techniques have been described for direct measurements of gludopa and its major catecholic metabolites other than DA and no studies have been performed on its pharmacokinetics.

As a DA prodrug, gludopa may have a role in hypertensive and sodium-retaining states. However, gludopa has been shown to be able to ameliorate glycerol-induced kidney tubular necrosis presumably through the action of DA [6] and as such may be useful as an antidote against drugs known to be nephrotoxic. We are presently evaluating gludopa as a means of reducing the tubular damage produced by the anti-cancer drug *cis*-dichlorodiamine platinum II (cisplatin, [7]). In this paper we describe a new, sensitive high-performance liquid chromatographic (HPLC) method for the determination of gludopa and its major metabolites L-dihydroxyphenylalanine (L-DOPA), DA and 3,4-dihydroxyphenylacetic acid (DOPAC). In addition, we describe an alumina-based extraction technique for plasma and urine and an ultrafiltration technique for tissues.

EXPERIMENTAL

Chemicals and reagents

DOPAC, 3,4-dihydroxyphenylglycol (DOPEG) and homogentisic acid (HGA) were from Aldrich (Poole, U.K.). Norepinephrine (NE), epinephrine (E), metanephrine, normetanephrine, homomandelic acid (HMA), vanillylmandelic acid (VMA), L-DOPA, DA and dihydroxybenzylamine (DHBA) were from Sigma (Poole, U.K.). Gludopa which was a gift from Professor Lee (Department of Clinical Pharmacology, Royal Infirmary of Edinburgh, U.K.), was synthesised by UCB Bioproducts (Brussels, Belgium) and was >98% pure peptide. γ-L-Glutamyldopamine (gluDA) was synthesised enzymatically from

dopamine and glutathione by γ-GT (Sigma) essentially according to the procedure described for gludopa [1]. Catechols were made up and diluted in 10 m*M* hydrochloric acid; they were stored at −20°C and renewed every month. Sodium dihydrogenphosphate (anhydrous, Aristar grade) and trichloroacetic acid (TCA, AnalaR grade) were from BDH (Poole, U.K.); methanol (HPLC reagent grade) was from Rathburn (Walkerburn, U.K.); heptanesulphonic acid (sodium salt, HPLC reagent grade) was from Pierce (Chester, U.K.) and EDTA was from Sigma. Water was deionised and bidistilled in a quartz glass still and all other chemicals were of the highest grade available commercially.

High-performance liquid chromatography

The liquid chromatograph consisted of a Waters Model 6000 pump, a Waters Wisp autosampler (Waters Assoc., Northwich, U.K.) or a Rheodyne injection valve (supplied by Crawford Scientific, Strathaven, U.K.) both set for a 20-μl injection volume, an ESA Model 5100A Coulochem electrochemical detector with a post-column Model 5021 conditioning cell (C) connected in series to a twin electrode (D_1 and D_2) Model 5011 high-sensitivity analytical cell (Severn Analytical, Sheffield, U.K.). Output from D_2 (the electrode last in the series) was connected to a potentiometric chart recorder set at an attenuation of 10 mV through a 10-mV line. After optimisation, detector voltages were fixed at +0.3 V for C, +0.1 V for D_1 and −0.3 V for D_2. Quantitation was by measuring peak heights in nA and then referring to calibration curves of standards run on the same day. Limit of detection was defined as the 3:1 ratio of peak height to baseline noise height. The analytical column was a 25 cm×4.6 mm I.D. stainless-steel column, packed with Spherisorb ODS-2 (5 μm particle size, Phase Separations, Deeside, U.K.). The mobile phase consisted of 50 m*M* sodium dihydrogenphosphate buffer adjusted to pH 2.9 with 1 *M* orthophosphoric acid containing 250 mg/l heptanesulphonic acid and 80 mg/l EDTA–methanol (90:10, v/v). The mobile phase was filtered through a 0.22-μm filter, vacuum-degassed and helium-degassed before use. Elution was performed at a flow-rate of 1.0 ml/min and at ambient room temperature.

Plasma and urine extraction

Plasma and urine were extracted using mini-columns prepacked with 10 mg of alumina and containing 1 ml of 1 *M* Tris–EDTA pH 8.6 (ESA plasma catecholamine kit, Severn Analytical). To 1 ml of plasma or urine was added DHBA as an internal standard in a volume of 50 μl. Samples were then added to the extraction columns which were capped at both ends and rocked for 10 min. Under positive pressure the alumina was washed twice with 2 ml of 1 *M* Tris–EDTA pH 8.6 solution. Gludopa and all other catecholic compounds were finally desorbed in 200 μl of 10% TCA and collected in polypropylene tubes.

Tissue sample preparation

Liver and kidney samples (approximately 0.5 g) were homogenised in two volumes (w/v) of 5% TCA using a standard laboratory blender. To 1 ml of homogenate was added DHBA in a volume of 50 μl. Samples were then filtered through a YMT membrane (molecular mass cut-off 30 000) contained in an MPS-1 micro-ultrafiltration unit (Amicon, Stonehouse, U.K.). The MPS-1 units were centrifuged at 1000 *g* for 30 min and the ultrafiltrates were collected in polypropylene tubes.

Analysis of samples

Blood immediately separated into plasma, kidneys and liver were collected from rats after 50 mg/kg gludopa as an intravenous bolus. Tissues were washed and all samples stored at $-20°C$ prior to analysis. In a separate experiment, urine was collected from rats in metabolic cages after the same dose of gludopa. Urines were acidified and stored at $-20°C$ prior to analysis. Urine was also obtained from patients receiving 9 mg/kg gludopa as a 6-h infusion in combination with a 1-h infusion of 100 mg/m^2 cisplatin.

RESULTS AND DISCUSSION

Electrochemical properties of gludopa

The aim of this present study was to develop an HPLC method which would be both specific and sensitive for gludopa and its catecholic metabolites. To achieve this we utilised the ESA coulometric dual-cell electrochemical detector, which is more sensitive for catecholamines than conventional amperometric electrochemical detectors [8,9]. Enhanced performance is due to the ability to oxidise or reduce interferences at the electrode surface and effectively eliminate these components from detection by downstream electrodes. Additionally, the analytical cell consists of two electrodes configured in series where one can perform oxidation whilst the other can perform reduction (or vice versa). Here, only compounds whose oxidation/reduction is reversible will be detected at the second electrode, thus introducing another element of selectivity. By choosing the optimal combination of conditioning cell and analytical cell potentials for the class of compounds of interest very high selectivity can be achieved. Catecholamines are well known for their facile two-electron reversible oxidation which makes them ideal candidates for electrochemical detection with a coulometric detector [8]. A hydrodynamic voltammogram of gludopa is shown in Fig. 2. Consistent with the behaviour of common catecholamines, the oxidation of the dipeptide was both facile and reversible. Peak height was much greater in the oxidation mode than the reduction mode, and at the monitor voltage of 0.3 V the oxidation/reduction (O/R) peak-height ratio was 3.33. Each catecholamine has a characteristic O/R peak-height ratio

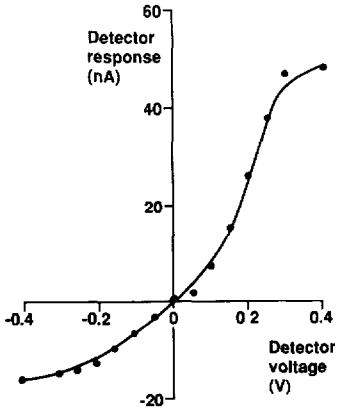

Fig. 2. Hydrodynamic voltammogram of gludopa. A constant amount (2 ng) of gludopa was repeatedly injected, the detector voltage (D_2) was varied and the detector response in the form of peak height in nA was recorded

and this has been used to determine peak purity, as electroactive co-eluting impurities are most likely to alter this ratio [10].

High-performance liquid chromatography

Capacity factors (k') for the HPLC separation are contained in Table I and a chromatogram of a mixture of standards of gludopa and its three major metabolites as well as NE, E and HGA (three potential interferences) is shown in Fig. 3. DOPEG, another potential interference, had a retention time (t_R) of 6.1 min and k' of 1.03 and chromatographed before NE. Retention times for all components varied by less than 7% throughout the day after equilibration with mobile phase for 1 h. Limit of detection for gludopa and its major metabolites injected directly on-column was: 10 pg for gludopa, 2 pg for L-DOPA, 4 pg for DA and 4 pg for DOPAC; plate number was >80 000 plates/m for each. Calibration curves for gludopa and its three major metabolites were all linear with $r > 0.999$ over a concentration range of 10 μg/ml to 1 ng/ml (200 ng to 20 pg injected on-column). The retention time of gludopa was found to be extremely pH-dependent: at pH 4.5 t_R was only 4.03 min, at pH 4.0 it was 5.75 min, at pH 3.5 it was 8.28 min, at pH 3.0 it was 12.9 min and at pH 2.75 it was 19.4 min. Thus, a 0.1 pH unit deviation from 2.9, the pH of choice, would result in a considerable drift in the t_R of gludopa and consequently pH of the mobile phase has to be carefully controlled. Only intact catechols which undergo facile reversible oxidation were detected by our electrode configuration; O-methyl metabolites such as HMA, VMA, metanephrine and normetanephrine, which do not undergo facile oxidation, were not detected.

In the past, organic phase gradient elution has been used along with a negatively charged ion-pairing agent to solve the problem of the large difference

TABLE I

HPLC AND SAMPLE PREPARATION OF γ-L-GLUTAMYL-L-DIHYDROXYPHENYLALANINE (GLUDOPA)

Chromatographic conditions and sample preparation techniques as described in Experimental.

Compound	Capacity factor[a] (k')	Retention time (min)	Plasma alumina extraction[b] (mean ± S.D.) (%)	Tissue homogenate ultrafiltration[c] (mean ± S.D.) (%)
L-DOPA	2.93	11.5	65.8 ± 4.2	87.5 ± 2.2
3,4-Dihydroxybenzylamine	3.76	14.3	58 0 ± 1.8	91.2 ± 7.3
Gludopa	5.16	18.5	60.3 ± 4.5	96.6 ± 2.2
Dopamine	6.13	21.4	58.5 ± 3.5	96.3 ± 3.2
3,4-Dihydroxyphenylacetic acid	7.00	24.0	60.2 ± 5.6	83.1 ± 4.2

[a]Within-day coefficient of variation less than 7% for all components.
[b]Control plasma (1 ml) spiked with 1 ng ($n = 25$) or 100 ng ($n = 25$) of each component. Values represent the mean from the combined study ($n = 50$).
[c]Control liver ($n = 10$) and kidney ($n = 10$) homogenates spiked with 100 ng of each component. Values represent the mean from the combined study ($n = 20$).

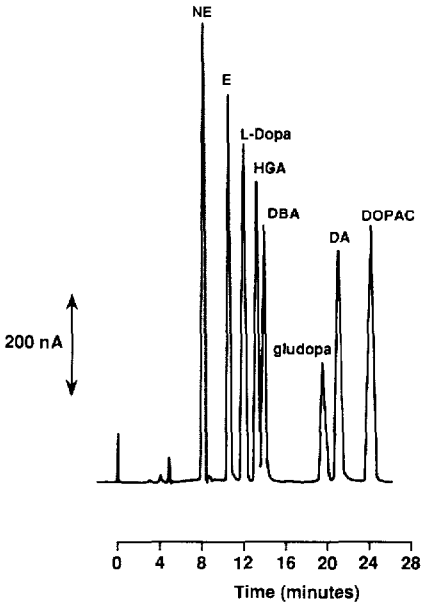

Fig. 3. Separation of 10-ng standards using the chromatographic conditions and detector voltages described in Experimental. Peaks: NE = norepinephrine; E = epinephrine; L-Dopa = L-dihydroxyphenylalanine, HGA = homogentisic acid; DBA = dihydroxybenzylamine; DA = dopamine; DOPAC = 3,4-dihydroxyphenylacetic acid.

in retention time between L-DOPA and DA [11]. This system caused considerable baseline drift during the gradient phase and precluded the use of DHBA as an internal standard. Also baseline drift occurred in the region where gludopa would be expected to elute. Subsequently, isocratic elution was used, em-

ploying a low percentage of organic modifier [12]. Here, L-DOPA had a t_R of approximately 8 min and DA had a t_R of >40 min and the efficiency was too low to enable measurement of endogenous levels of DA. We have used a high percentage of organic phase to achieve high efficiency (>80 000 plates/m) but have modified the pH of the aqueous phase to increase retention specifically of L-DOPA, DOPAC and especially gludopa in order to reduce the difference in t_R between DA and shorten run time (see Table I). The separation is free from interference from the commonly occurring catecholamines NE and E and their O-methyl metabolites, as well as DOPEG.

Sample preparation

Alumina is known to extract L-DOPA and especially DOPAC with low and variable efficiency from plasma [13]. In preliminary experiments we investigated different eluting solutions (acetic acid versus TCA) and ionic strengths (1%, 5% and 10% TCA) to improve on these low efficiencies and to optimise for gludopa. We found that 10% TCA was required for good and reproducible recoveries. Acetic acid (4%) which is the normally recommended eluting solution for ESA pre-packed alumina columns, only desorbed gludopa with approximately 20% efficiency. Samples stored in 10% TCA could be frozen to −20°C, thawed and re-analysed without any significant loss of component of interest. Samples could also be kept at room temperature for up to 24 h without significant loss and after one week at room temperature approximately 10% reduction in peak height occurred. In control experiments, either 1 or 100 ng of gludopa, DHBA, L-DOPA, DA and DOPAC were each added to 1 ml of blood blank plasma. Mean recoveries from both studies ($n=50$) are shown in Table I and were almost identical for all five components.

Alumina extraction of urine or plasma for catecholamine analysis has been criticised for not producing sufficiently clean samples for unambiguous quantitation [9]. Alternatives that have been suggested either require the use of a combination of solid-phase materials [13] or complicated procedures involving a single solid phase such as silica bonded with a strong cation-exchange group [14], boric acid gels [15] or silica bonded with phenylboric acid [13]. Alumina on its own has been shown to be sufficient when (a) combined with a high-efficiency HPLC separation and (b) coupled with a coulometric detection system by which selectivity can be improved [16]. Whilst two-stage procedures do clean up specimens they have also been shown to lose DA [17], which is the essential element in the present analysis. We have chosen a rapid and simple alumina-based technique which extracts gludopa, its major metabolites and DHBA with consistent and good recoveries.

When kidneys and livers were homogenised in phosphate-buffered 0.9% sodium chloride we discovered that such tissue homogenates retained the ability to metabolise gludopa, especially in the case of the kidney. Thus, tissues were homogenised in 5% TCA to denature proteins and stop enzyme activity. Ultra-

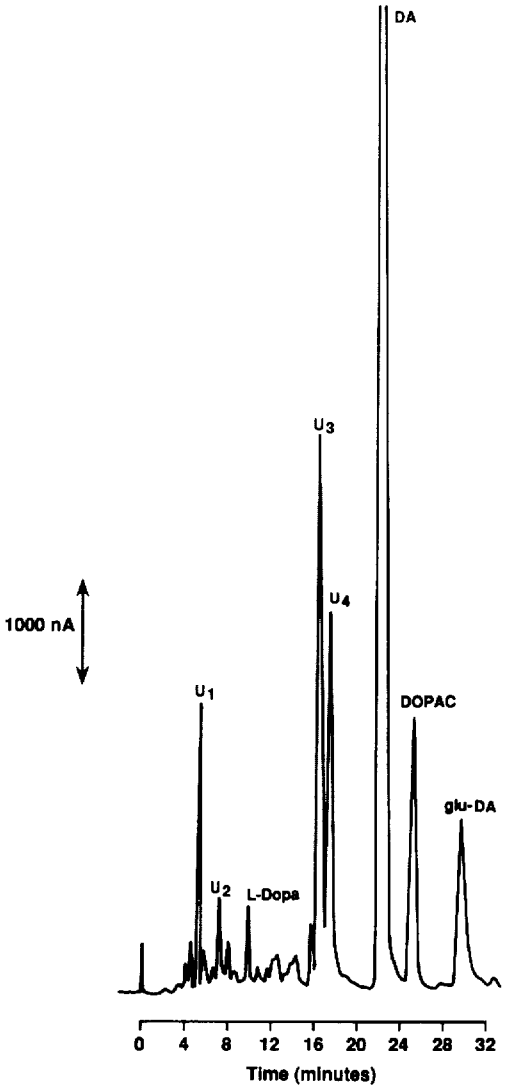

Fig. 4. Analysis of 1 ml urine from a rat treated with 50 mg/kg gludopa intravenously. Four major metabolites of gludopa were identified: L-DOPA, 0.39 μg/ml; DA, 45.1 μg/ml; DOPAC, 2.2 μg/ml; gluDA, 1.3 μg/ml. Four other major metabolites were detected (U_{1-4}) and at least twelve 'minor metabolite' peaks were evident.

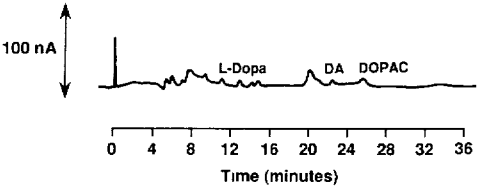

Fig. 5. Analysis of urine from a non-gludopa-treated rat run at ten times the sensitivity of Fig. 4. Trace amounts of L-DOPA, DA and DOPAC were detected but it is clear that the 'minor metabolite' peaks of Fig. 4 are not present.

filtration resulted in very little loss of all components of interest. Recoveries after addition of 100 ng ($n=20$) of gludopa, DHBA, L-DOPA, DA and DOPAC to control liver and kidney homogenates are shown in Table I.

Analysis of samples

In urine of rats treated with 50 mg/kg gludopa (similar to the dose effective in ameliorating glycerol nephrotoxicity) up to eight major metabolite peaks were resolved, four of which could be identified against standards. Further

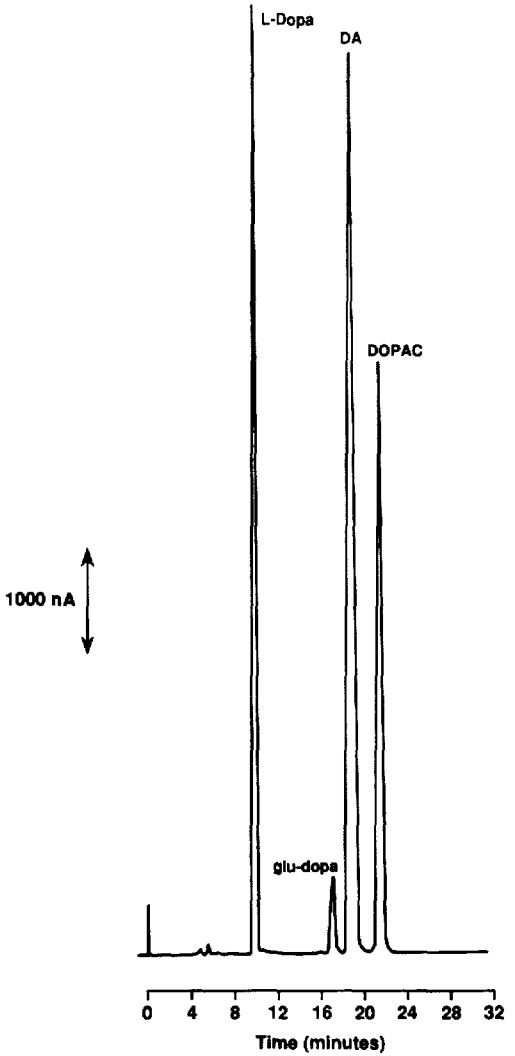

Fig. 6. Analysis of urine from a patient treated with 9 mg/kg gludopa intravenously. Peaks are: L-DOPA, 12.6 μg/ml; gludopa, 2.8 μg/ml; DA, 19.5 μg/ml; DOPAC, 11.8 μg/ml.

minor metabolite peaks were also apparent (compare Fig. 4 against Fig. 5). No gludopa was detectable and the level of DA was far in excess of any other species measured (Fig. 4) accounting for 85% of total excreted catecholic species. By comparison, in human urine from patients treated with gludopa, 9 mg/kg, the compound was clearly detectable and only its three metabolites were present, in this case in roughly equal amounts (Fig. 6).

The rat tissues (liver and kidney) contained only L-DOPA, DA, DOPAC and gluDA as well as gludopa at levels which were detectable for up to 4–6 h after drug administration. Thereafter their concentrations fell to the range 1–10 ng/g and interference from endogenous substances became a problem. The higher limit of detection is probably due to the fact that tissues have only been deproteinated by filtration and not extracted on alumina.

Several analytical techniques have been employed to study the conversion of gludopa specifically and only to DA: these include gas chromatography for tissue [1], gas chromatography–mass spectrometry in isolated kidney perfusate [18], radioimmunoassay in plasma and urine [5] and recently HPLC with amperometric electrochemical detection for urine [19]. In none of these studies was gludopa measured, nor any of its other major catecholic metabolites, such as L-DOPA or DOPAC. Nor had pharmacokinetics been performed. If gludopa is to be used as an antidote in drug-induced kidney toxicity then it is important that its pharmacokinetics in the target organ are well understood in order that the correct drug schedule is administered to ensure maximum conversion to DA at the time when the toxic agent is present in the kidney at its highest concentration. We have developed a new HPLC method with electrochemical detection for the measurement of this compound. The sample preparation and HPLC techniques allow the simultaneous determination of gludopa and its major metabolites L-DOPA, DA and DOPAC in plasma, urine and tissues. Presently, this method is being applied to study the pharmacokinetics and metabolism of gludopa in both humans and experimental animals.

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