Analysis of L-Dopa in Human Serum

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Among the drugs acting on the dopaminergic system, L-dopa is the most important one for treatment of patients with Parkinson's disease (2). The monitoring of L-dopa concentrations in plasma has clarified and solved several pharmacokinetic problems, such as recognizing pharmacokinetic interactions and producing new types of preparations such as controlled release drugs (2).

Sagar and Smyth (3) recently published an advanced column-switching method for the analysis of L-dopa and referenced 18 methods from 1962–1986, but not more modern and simpler methods. In studies of the pharmacokinetics of the drug (1), we used an HPLC method combined with a simple clean-up procedure employing the properties of boronate gel to capture chemical compounds that, similar to L-dopa, contain vicinal hydroxyls. After washing the sample adsorbed to the gel, L-dopa was eluted in a weakly acidic solution and subjected to HPLC. However, the boronate affinity gel PBA-30 (Amicon, Danvers, MA, USA) we used disappeared from the market, and in search for a substitute, we found a similar preparation, Prosep®-PBA (Millipore, Consett, Durham, UK), which was suitable for our purposes. We also investigated the boronate binding of α -methyldopa as a promising internal standard candidate.

We used L-dopa from Merck (Darmstadt, Germany) and α -methyldopa from Sigma (St. Louis, MO, USA). We prepared 1 mL boronate gel in Econo-columns (0.7 $\times$ 4.0 cm; Bio-Rad Laboratories, Hercules, CA, USA) by equilibrating the gel with 25 mL 100 mM phosphate buffer, pH 8.3, containing 2.7 mM EDTA and 5.3 mM sodium metabisulphite. The columns could be re-equilibrated and reused several times.

Stock solutions of L-dopa and DL- α -methyldopa (both 10 mM) were prepared in an aqueous solution of 10 mM hydrochloric acid and 0.2 mM EDTA. These solutions were freshly prepared on the day of analysis. We prepared a working standard solution by diluting L-

dopa and α -methyldopa to 2.0 μ M with mobile phase (0.1 mM formic acid, 0.2 mM EDTA, pH 3.1) and made solutions with varying concentrations of the calibrator and internal standard for experimental studies. The α -methyldopa was diluted to 100 μ M with the hydrochloric acid-EDTA solution and added to the samples as an internal standard.

For the analysis of L-dopa, we added 1.3 mL serum sample to analytical tubes. Then 26 μ L 100 μ M α -methyldopa were added, the samples were thoroughly mixed, and 35 μ M EDTA were added and mixed. For protein precipitation, 200 μ L 4 M HClO₄ were added, samples centrifuged at 3000 $\times$ g at 4°C for 10 min, 1.1-mL aliquots of the supernatants were added to new tubes containing 1.3 mL 1 M K₂HPO₄, and the samples were thoroughly mixed. The samples were centrifuged at 3000 $\times$ g at 4°C for 10 min, and 2.2 mL of the supernatants were transferred to the equilibrated Prosep-PBA boronate columns. The column was washed with 1.5 mL 100 mM phosphate buffer, pH 8.3, containing 2.7 mM EDTA and 5.3 mM sodium metabisulphite. For elution of L-dopa and α -methyldopa, 0.75 mL 100 mM phosphate buffer, pH 2.3, and 0.2 mM EDTA were added. This fraction was discarded. Then, 1 mL of the same acid buffer was added, and the fraction was collected in plastic vials for analysis by HPLC. The sample injection volume was 20 μ L.

The equipment used was a model 590 HPLC pump from Waters (Milford, MA, USA), an automatic sample injector model 231 XL from Gilson (Middleton, WI, USA), and an electrochemical detector model 460 from Waters. A model 740 data module (Waters) was used for integration and calculations.

The analytical column was a Supelcosil C₁₈ column (250 $\times$ 4.6 mm i.d., particle size 5 μ m) from Supelco (Bellefonte, PA, USA). The temperature was maintained at 30°C by a Temperature Control Module thermostat and column heater (Waters).

The mobile phase was a solution of 0.1 mM formic acid and 0.2 mM EDTA with pH adjusted to 3.1 with sodium hydroxide. The solution was filtered through a 0.45- μ m cellulose acetate filter (Sartorius, Göttingen, Germany).

Venous blood samples were ob-

tained from Parkinson's patients on treatment with L-dopa. Blood samples were collected in 10-mL vacuum tubes without additives (Becton Dickinson). After coagulation, the samples were centrifuged at 3000 $\times$ g at 4°C for 10 min within 4 h of collection. Serum was then stored at -70°C until analysis.

The L-dopa and α -methyldopa peaks were well separated on the chromatogram with retention times of 7.8 and 15.4 min, respectively. The detector responses were linear over the range of 0–10 μ M for both substances. When the serum samples were analyzed, an unknown peak appeared at 9.3 min. This peak was also well separated from L-dopa.

We investigated the optimal equilibration pH for the Prosep-PBA column before loading the sample. The sample was prepared from serum spiked with both L-dopa and α -methyldopa. Very

Figure 1. Chromatogram of serum L-dopa from a patient with Parkinson's disease. After continuous intravenous infusion of 85 mg L-dopa/h into the patient, a sample was obtained from the contralateral arm. The concentration of L-dopa was 3.26 μ M.

Benchmarks

Table 1. Results of Duplicate Analyses of 23 Samples Obtained from Patients with Parkinson's Disease on L-Dopa Therapy

Number of Samples (n)	Range (μM)	$\bar{x} \pm \text{sd}^a$ (μM)	cv (%)
6	0–0.2	0.072 ± 0.010	14
8	0.2–4.0	1.98 ± 0.038	1.9
9	4.0–9.0	6.13 ± 0.058	0.95

Results were stratified into groups with low, medium, and high values. ^aCalculated within each group from the squared difference between duplicates.

small amounts (<10%) of L-dopa or α -methyldopa were found in the first 0.75-mL wash, but the next 1-mL fraction was purified from the peak with a retention time of 9.3 min and contained the majority of L-dopa and α -methyldopa. With the columns pre-equilibrated at pH 8.0–8.6, the recoveries in this fraction were 79.3%–80.4% for L-dopa and 78.5%–79.6% for α -methyldopa (see below for calculation). The mean difference in recoveries between the compounds was 1%. We chose to pre-equilibrate the columns at pH 8.3, which was slightly higher than was used earlier (pH 8.0) with the boronate affinity gel PBA-30 (1).

From control serum, we prepared a pool containing 2.0 μM L-dopa and 2.0 μM α -methyldopa. Five replicates of this pool were used for protein precipitation and further purification on the boronate column. The concentration of L-dopa found in the eluate was $1.19 \pm 0.03 \mu\text{M}$ (cv 2.5%), and the α -methyldopa concentration was $1.24 \pm 0.07 \mu\text{M}$ (cv 2.9%), as calculated using the respective standard curves. Theoretically, according to the volume changes in the precipitation steps, 58% of the original sample was transferred to the column (e.g., $0.58 \times 1.3 \times 2 \text{ nmol} = 1.51 \text{ nmol}$). At 100% recovery across the boronate purification step, an eluate concentration of 1.51 μM should thus be expected. This means that the absolute recoveries across the boronate columns were 79% and 82% or better for L-dopa and α -methyldopa, respectively. The quotient between the compounds in the five eluates was $0.94 \pm 0.013 \mu\text{M}$ (cv 1.4%). Thus, it seems that the recovery and the imprecision of the method were very satisfactory and the imprecision was even better if α -methyldopa was

used as an internal standard.

Table 1 shows the imprecision of the method as calculated from samples analyzed in duplicate using α -methyldopa as internal standard. At therapeutic L-dopa concentrations, the cv was less than 2%. From the samples with very low values, the detection limit of the method could be calculated. A detection limit of 0.03 μM was obtained using three standard deviations as the definition of detection limit. This is as good as the data given by Sagar and Smyth (3) using a complicated column-switching technique and EC detection.

The technician's time for pre-purification of one sample is 1 h, and 20 samples can be processed within 4 h. The chromatographic time is 20 min/injection. The costs of the Prosep-PBA gel is \$10/sample, and we have regenerated the Prosep-PBA columns for reuse five times without problems. This reduces the costs further. The other reagents are very inexpensive. Thus, we think that this method is cost-effective. One may argue that the protein precipitation step might be possible to eliminate. Therefore, we tried to omit this step by only adjusting the sample pH to 8.3 before loading to the Prosep-PBA column. However, most proteins were retained on the boronate column and eluted together with the L-dopa. Therefore, the initial protein precipitation step seems necessary.

In extensive pharmacokinetic studies, many samples are collected and the amount of blood taken for analysis should be kept low. In this situation, the method can easily be scaled down to, for example, 0.5 mL serum or less, since the analytical sensitivity is so high. Still, lesser amounts of serum can be used because we only used 2% of

the eluate from the boronate step for HPLC, and the injection volume therefore can be increased.

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