Quantitation by Gas Chromatography-Chemical Ionization-Mass Spectrometry of Phenylalanine Mustard in Plasma of Patients

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ABSTRACT

An unambiguous and sensitive method based on gas chromatography-chemical ionization-mass spectrometry has been developed to quantitate L-phenylalanine mustard and has been applied to measure levels in plasma of five patients receiving 0.15 to 0.25 mg/kg (10 to 17 mg) of the drug p.o. Peak plasma levels of 50 to 190 ng/ml were found to occur between 0.7 and 2.3 hr after ingestion. The time for the plasma level to fall to one-half of the peak value varied from 0.6 to 3 hr, and very low levels (<2 ng/ml) were present by 24 hr.

INTRODUCTION

L-PAM (Chart 1) has been used clinically since the late 1950's in the treatment of ovarian cancer, myeloma, and breast cancer. It has been administered i.v. (mostly in Europe) and p.o. Intermittent i.v. doses of L-PAM of up to 1 mg/kg have been used, but p.o. regimens of 0.025 to 0.1 mg/kg daily or 0.15 to 0.25 mg/kg/day for 4 to 5 days every 4 to 6 weeks have been most widely used.

In most of the published studies of the human pharmacology, radioactive L-PAM has been administered, and blood levels of radioactivity have been determined by techniques which do not distinguish between parent compound, metabolites of similar lipophilicity, or the products of spontaneous chemical hydrolysis. In one previous study of the pharmacokinetics of radioactive L-PAM in patients (8), the total plasma radioactivity was found to peak between 1 and 3 hr after p.o. doses. These authors found that patients who received the drug p.o. excreted less label in their urine than did patients receiving the drug i.v., and they concluded that absorption is incomplete following p.o. administration. A pharmacokinetic study after p.o. administration in dogs has also been reported (6). In this study, total plasma radioactivity was measured by scintillation counting, while the parent compound was assayed with unspecified sensitivity by a combination of high-pressure liquid chromatography and scintillation counting.

High-pressure liquid chromatography has been proposed as an analytical technique (3, 6) to be used in addition to or instead of scintillation counting to provide compound-specific identification. In one paper, sensitivity has been reported to be as good as 50 ng/ml plasma using high-pressure liquid chromatography (3). However, these techniques do not correct for spontaneous decomposition during the workup (2). Assays with limited sensitivity using gas chromatography (7) and direct-probe mass spectrometry (8) have also been reported.

In view of the absence of compound-specific measurements at levels below 50 ng/ml plasma in patients receiving p.o. doses of phenylalanine mustard, and in order to approach detailed pharmacokinetic analyses of this drug, we set out to develop a gas chromatographic-mass spectrometric assay. We have exploited the sensitivity and specificity of selected ion monitoring to permit evaluation of blood levels in patients receiving conventional p.o. doses of L-PAM. Mass spectrometric detection also permits use of a stable isotope-labeled internal standard in order to correct directly for reversible protein binding, spontaneous sample decomposition, and sample losses in extraction and derivatization.

We have applied these methods to measure the levels of L-PAM in the plasma of 4 patients receiving 0.15 to 0.25 mg of the drug per kg and to a patient receiving a similar dose immediately before undergoing hemodialysis.

MATERIALS AND METHODS

Descriptions of the patients and the doses of L-PAM that they received are given in Table 1. Each patient received the indicated dose of L-PAM daily for 4 days. All patients except Patient 2 were studied on the day of the first dose. Patient 2 was studied on Day 2 of drug administration. Patient 5 had severe renal failure and was maintained on hemodialysis. The only patient receiving additional medication was Patient 5, who was given prednisone at the time of dialysis. On the day of the study, hemodialysis was begun immediately after L-PAM administration and was continued for 4 hr during the time when the plasma samples were being obtained. An attempt was made to assay L-PAM levels in the dialysate fluid, but high levels of an interfering compound (probably prednisone) in the dialysate fluid prevented this analysis.

At specific intervals, patient blood was drawn directly into heparinized tubes and centrifuged, and the plasma was immediately flash frozen with dry ice-acetone. Prior to the assay, the plasma was thawed in lukewarm water and 2-ml aliquots removed. An internal standard of 100 ng DL-PAM-d8 was added to each ml of plasma, and the spiked solution was refrozen until use. DL-PAM-d8 was synthesized from racemic phenylalanine following the procedure of Bergel and Stock (1), using ethylene-d2 oxide (98 atom % deuterium) from Merck and Company, Inc., Rahway, N. J.

The plasma sample with its internal standard was thawed...
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and then immediately precipitated with an equal volume of 5% perchloric acid and centrifuged. The supernatant was removed and stored on ice. The precipitate was washed with 1 ml of 5% perchloric acid solution and recentrifuged. The supernatants were combined. The cold combined supernatant was percolated through a thiamine assay chromatography tube (Arthur H. Thomas Co., Philadelphia, Pa.), 125 mm long x 6 mm inside diameter, which had been packed with prewashed (methanol and water) Porapak Q (50 to 80 mesh) (Waters Associates Inc., Rochester, Minn.). The column was washed with 12 ml of cold distilled water, and phenylalanine mustard was removed with 8 ml of cold methanol (Burdick and Jackson Labs., Inc., Muskegon, Mich.). The methanol, which contained some water, was eluted directly into a 25-ml pear-shaped boiling flask and immediately evaporated to complete dryness.

The dried sample was acetylated with trifluoroacetic acid: acetoni-trile (1:1) for 20 min at room temperature under a nitrogen atmosphere. This solution was blown dry with nitrogen, and an ethereal solution of diazomethane (generated from Aldrich Diazalod) was added and was allowed to react for 20 min. The solution containing the derivatized product was transferred to a reacti-vial (Pierce Chemical Co., Rockford, Ill.) and blown dry with nitrogen. The residue was stored in the freezer. Just before mass spectrometric analysis, the samples were reconstituted with 20 or 30 μl of silylation grade acetonitrile (Pierce Chemical Co.). In preparation of the calibration curves, standard mixtures of undeuterated and deuterated material were added to normal plasma. An internal standard of 100 ng DL-PAM-d8/ml plasma was used in conjunction with a range of L-PAM-d0 of 0 to 200 ng/ml plasma. Precipitation, extraction, derivatization, and mass spectral analysis were carried out in parallel with the patient samples.

A DuPont 321 gas chromatograph-mass spectrometer computerized with Riber software was used for selected ion monitoring and quantitation at 0.1 amu specificity. The samples were chromatographed on a 3% OV-101 80 to 100 mesh Supelcoport 5 foot x 2-mm (inside diameter) glass column with temperature programming at 210–280° at 8°/min. Instrumental parameters included jet separator temperature at 250°, injector at 280°, mass spectrometer source at 200°, and helium flow of 20 ml/minute through the gas chromatograph. Injections were done in triplicate, and the first 2 min of each sample run was ‘‘dumped’’ via a diverter valve to preserve filament life and cut down on the source contamination which would have lowered sensitivity. Chemical ionization with isobutane gas was used throughout the study. Selected ion monitoring was carried out by first calibrating the mass spectrometer with perfluorotributylamine and then tuning to the top of the M + H peak of derivatized phenylalanine mustard, thus obtaining the 0.1 amu accurate mass measurement. In the assay, the M + H ions of both L-PAM-d0 and DL-PAM-d8 were recorded correct to 0.1 amu, along with the ion current 0.1 amu higher. The latter reading monitored any instrumental mass drift over the analysis period. The parameters selected provided a 4-channel record with a cycle time of 1 sec. Ratios of computer-integrated peak areas [m/z 415.0]/[m/z 423.1] and manually measured peak heights were found to be very similar. Measurements of peak heights were made on both standard curves and patient samples throughout the study. Calibration curves which related ion intensity peak height ratios to phenylalanine mustard concentration were linear over a range of <2 to 200 ng/ml of plasma. Data from 6 points on each curve with triplicate injections at each point were processed by linear regression. New standard curves were determined each time a set of patient samples was run.

RESULTS

Several different protocols were evaluated for derivatization, including dimethyilsilylation with bis(trimethylsilyl)trifluoro-acetamide (3, 5), esterification with methanol and hydrochloric acid, methylation with dimethyformamide dimethylacetel (9), treatment with diazomethane followed by N-trifluoroacetylation, and the route ultimately adopted, trifluoroacetylation followed by esterification with diazomethane. This last protocol was selected by comparing recoveries, sample stability, and signal: noise ratio in selected ion profiles of the corresponding protonated molecular ions. Dimethyilsilylated derivatives provided a mass spectral signal equally as intense (chemical ionization); however, the sample decomposed completely when stored in
silylating reagent for 24 hr. The dimethylformamide dimethyl-acetyl derivative gave protonated molecular ions only one-sixth as abundant as those of the N-trifluoroacetyl methyl ester.

Radioactive L-PAM added to plasma (100 ng/ml) was used to compare recoveries in methanol elutions of Porapak Q and XAD-2. Recovery from the former was 92 to 95% and recovery from the latter was 73 to 78%. Radioactive material (19 to 20%) was also eluted from XAD-2 in the water wash. However, decomposition of the parent compound was extensive in this aqueous solution. Recovery of unaltered parent compound from plasma in the methanol wash from Porapak Q was confirmed at 90% by derivatization and gas chromatography-mass spectrometry. This coincidentally provides an evaluation of the efficiency of our entire protocol including extraction, derivatization, and gas chromatography-mass spectrometry analysis.

Concern about the possible loss of the parent compound by hydrolysis in the aqueous perchloroacetate solution used to precipitate protein led us to evaluate decomposition in this solution. Using the gas chromatography-mass spectrometry assay, we found that 40 to 45% of the drug was hydrolyzed in 1 hr. Consequently, samples were processed individually or in pairs to complete this part of the protocol as rapidly as possible. This rate of hydrolysis is similar to that reported by Chang et al. (2) for other kinds of aqueous solutions.

Selectivity and signal/noise ratios were improved by several variations in the gas chromatography-mass spectrometry methodology. Partial mass spectra of derivatized L-PAM-d0 obtained using electron-impact ionization and chemical ionization are shown in Charts 2 and 3. The M + H peak at 415 has a relative intensity of 100% in chemical ionization, while the M* peak has a relative intensity of only 20% in electron impact.

The molecular ion region of the chemical ionization isobutane spectrum shown in Chart 4 clearly demonstrates the characteristic pattern of a 1:1 mixture of L-PAM-d0 and DL-PAM-d8 N-trifluoroacetyl methyl esters, both of which contain 2 chlorines. The protonated molecular ions with their corresponding isotope species in a 9:6:1 ratio are at m/z 415, 417, and 419 and at m/z 423, 425, and 427, respectively.

The nonselective detection method of total ion chromatography cannot be used for the quantitation of L-PAM, as levels of 200 ng/ml plasma (combined L-PAM-d0 and DL-PAM-d8 analogs) are not reliably detected above the signal level of interfering plasma compounds, even when utilizing chemical ionization. Chart 5 shows ion currents integrated in the narrower range between m/z 410 and m/z 430 using both electron impact and chemical ionization. When chemical ionization is used, a peak is clearly visible at the retention time of the combined L-PAM-d0 and DL-PAM-d8 analogs, indicated by the arrows. This peak corresponds to a combined level of 200 ng/ml plasma or approximately 24 ng injected.

In order to achieve sensitivity in the low ng/ml range or better, selected ion monitoring was required. Real-time profiles of ion current at m/z 415.0 (M + H)+ for L-PAM-d0 N-trifluoroacetyl methyl ester and m/z 423.1 (M + H)+ for derivatized PAM-d8 are shown in Chart 6. The sample injected was an...
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A typical standard curve is shown in Chart 7. The linearity of the curve attests to the precision of the protocol. The curve does not cross the abscissa at zero because a small amount of signal is present at 415 in the absence of L-PAM-d₆. Correction is made for this in all the clinical assays. In a set of 11 injections of sample prepared from plasma spiked with L-PAM (100 ng/ml) and DL-PAM-d₈ analog (100 ng/ml) and equivalent to 24 ng combined L-PAM-d₆ and DL-PAM-d₈/injection, the mean peak ratio was measured as 1.023 ± 0.016 (S.D.). Standard curve data were analyzed and plotted by the method of linear regression. The coefficient of variation and correlation coefficient were also calculated. Chemical ionization selected ion monitoring is sensitive in our hands to less than 500 pg of pure derivatized drug, and the entire protocol reported here permits analysis of concentrations in plasma as low as 2 ng/ml with reproducible accuracy and precision.

Curves of plasma concentrations as a function of time for 5 patients are shown in Chart 8. The highest plasma concentrations of L-PAM in these patients were seen between 1 and 2.5 hr. The peak levels were between 50 and 190 ng/ml, and increased with increasing dose. The time for the plasma levels to fall to 50% of the peak levels varied between 36 and 180 min. At 24 hr, levels had fallen below 2 ng/ml in the 3 patients for whom samples were available.

DISCUSSION

Tattersall et al. (8) administered radioactive L-PAM to patients both i.v. and p.o. After i.v. administration, there was an initial rapid decline of radioactivity followed by a prolonged decay phase with a t₁/₂ of 160 hr. After p.o. administration of doses similar to those used in our study, peak plasma radioactivity levels occurred at 1 to 3 hr after the dose, followed by a slow decline similar to the terminal phase after i.v. administration. In one patient given i.v. L-PAM, plasma levels of the parent compound were determined and a t₁/₂ of 67 min for the drug was estimated. Peak levels of total drug equivalents seen in these patients were between 50 and 150 ng/ml.

Chang et al. (3) measured plasma concentrations of L-PAM by an HPLC technique sensitive to 50 ng/ml. In patients given 0.6 mg/kg either i.v. or p.o., t₁/₂ β values of 121 ± 99 and 211 ± 191 min were found for i.v. and p.o. administration, respectively.

Our data for p.o. doses of 0.15 to 0.25 mg/kg are consistent with both of the above reports. Both our results and those of Chang et al. (3) indicate that the prolonged levels of plasma radioactivity, seen by Tattersall et al., were due to hydrolysis products and metabolites rather than the parent compound.

The rapid decline in plasma levels and absent or low levels seen toward the end of the 24-hr period could suggest (at least, at these doses) that significant accumulation of the drug, with repeated daily doses, does not occur. This suggestion is supported by the data from Patient 3 who was studied after the second daily dose of L-PAM.

The information on Patient 5 is of interest in that it was similar to that from the other patients, despite the fact that the patient was on hemodialysis for the 4 hr following drug administration. This information suggests that the drug is not removed from the plasma to a significant degree by hemodialysis. This finding
might be expected from the high degree of protein binding which has been reported by others (2, 4).

The analytical method reported here includes some suggestions for extraction and derivatization which may be useful with assay techniques other than selected ion monitoring, including high-pressure liquid chromatography and gas chromatography. Selected ion monitoring by gas chromatography-mass spectrometry provides outstanding selectivity, specificity, and sensitivity. However, it is expensive, labor is intensive, and it has a low throughput. We are currently adapting our protocols for gas chromatography using a nitrogen detector.

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REFERENCES

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