

# Compliance Testing in Hypertensive Patients based on LC-MS-MS Measurement of Amlodipine Analytical Unit & \*Blood Pressure Unit St Georges - University of London, London, UK

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## INTRODUCTION

Whether it be due to unwanted side effects, concern over safety, attention seeking behaviour or just pure forgetfulness, medication non-compliance (adherence) is a significant issue doctors have to contend with, and its investigation can be both time consuming and costly. The Forensic Toxicology Service was approached as a last resort, following numerous investigations into patients that were resistant to their anti-hypertensive therapy. In each instance the patient was insistent that they were compliant despite no objective improvement in the control of their blood pressure. In response to the request, we developed methodology to determine amlodipine concentrations in patients apparently resistant to therapy.

Amlodipine, otherwise known as Istin® or Norvasc®, is a third generation dihydropyridine (DHP) calcium channel blocker, belonging to the same family as lercanidipine and lacidipine. It functions by selective inhibition of calcium influx across cell membranes, resulting in a greater effect on smooth than cardiac muscle cells. It is indicated for the treatment of hypertension and angina. Amlodipine is available in tablet formulation containing 2.5, 5 and 10mg doses of active drug. Amlodipine has a bioavailability of between 64 and 80% and peak plasma concentrations are between 6 and 12 hours after dosing. Steady state plasma concentrations are achieved following 7 to 8 consecutive days of dosing.



**Figure 1.** 10mg Amlodipine tablets: White emerald shaped with AML-10 imprinted on one side and the Pfizer logo on the other.

Amlodipine is highly protein bound (98%) and has a plasma half-life of 35 to 50 hours, longer in the elderly or those with hepatic impairment. This low metabolic clearance lends itself to once daily administration. The slow onset, prolonged duration of action and long plasma half life are typical of third generation DHPs. Amlodipine is slowly but extensively metabolised to inactive metabolites by the liver via the cytochrome P450 system, predominantly the CYP 3A4 isoenzyme. Plasma concentrations are therefore influenced by CYP 3A4 inhibitors such as ketoconazole. Less than 10% of the parent drug is eliminated by urinary excretion.

## CASE STUDY

We describe here an LC-MS-MS method for amlodipine compliance testing in patients with apparently refractory hypertension. One patient was referred to our clinic following thorough investigations at 3 other centres. Her sitting blood pressure was 190/121 mmHg and her heart rate was 96/min, despite therapy with maximum doses of multiple drugs including atenolol (50 mg bd), diltiazem (180 mg bd) and amlodipine (10mg bd). None of these drugs were detected in her plasma apart from amlodipine. The patient had no explanation for these findings and failed to return for follow up.

## EXPERIMENTAL

### Materials

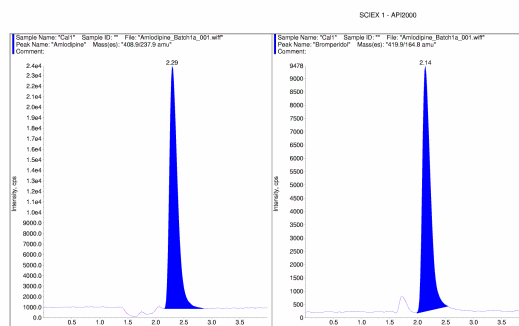
A pure reference standard of amlodipine was kindly provided by Pfizer (Sandwich, England). The internal standard, bromperidol was obtained from Janssen Pharmaceutica N.V. (Belgium). HPLC grade methanol and methyl-tert-butyl-ether (MTBE) were purchased from Rathburns Chemicals Limited (Walkerburn, Scotland). AnalaR grade formic acid was obtained from BDH (Poole, Dorset, England). Trisma base was purchased from Sigma (Steinheim, Germany). De-ionised water was prepared on site (ELGA Limited).

### Extraction

Amlodipine was extracted using liquid-liquid extraction. Stock solutions were prepared in methanol and all sub-stocks and standards were prepared in 50% methanol, owing to the drugs poor solubility. To 100µL of standard or sample (plasma) were added; bromperidol, 250µL of 2M Trisma base and 2mL MTBE. The samples were mixed and the phases separated by centrifugation. Amlodipine and the internal standard were back extracted from the organic phase into 250µL of 1% formic acid. The formic acid extracts were transferred to autosampler vials and left to stand for 5 minutes to allow any residual MTBE to evaporate, prior to analysis. 25µL of extract was injected onto the LC-MS-MS.

### HPLC Conditions and MS Parameters

The HPLC equipment consisted of a Perkin Elmer PE200 series autosampler and pump and a Shimadzu CTO-10A column oven. Detection was by tandem mass spectrometry (LC-MS-MS), using a Sciex API2000 triple quadrupole mass spectrometer equipped with a turbo-ion spray interface (Applied Biosystems). The analytical column used was an Alltech, Alltima C18 (150mm x 2.1mm, 5µm) and the mobile phase consisted of 80% methanol supplemented with 5mM ammonium formate. The method was run in positive ionisation mode and set to detect the precursor and product ion transitions of: amlodipine ( $m/z$ : 409/238) and bromperidol ( $m/z$ : 420/165). Amlodipine and bromperidol eluted after 2.29 and 2.14 minutes, respectively.



**Figure 2.** Representative chromatogram of a 100ng/mL plasma amlodipine calibrator and the internal standard, bromperidol.

## RESULTS AND DISCUSSION

### Validation

The method was linear from 2.5ng/mL to 100ng/mL. Calibration curves were calculated using  $1/x^2$  weighted regression through zero. The regressions coefficient  $r^2$  was  $>0.99$  ( $n=3$ ). The within and between assay precision (repeatability) and the accuracy were calculated for the LLOQ, ULOQ and the three QC's (4ng/mL, 40ng/mL, 75ng/mL). Each were analysed six times, in three separate assays, on three separate days. The within assay precision for all was  $<12.1\%$  and the between assay precision for all was  $<13.9\%$ . Accuracy ranged between 93.9% and 97.1%. Absolute recovery was evaluated in triplicate at each QC concentration and averaged 69% across the calibration range. There were no significant matrix effects observed for six different sources of plasma tested (maximum bias of +6.8%), and no interfering compounds at the retention time of the analyte were observed for the analyte-free plasma samples run with each calibration curve.

In a control population of patients on a once daily 5mg ( $n=9$ ) or 10mg ( $n=8$ ) dosage the mean plasma random amlodipine concentrations were 6.9ng/mL (3.2-12.8ng/mL) and 14ng/mL (4.9-24.9ng/mL) respectively. Mean plasma concentrations of 2.0ng/mL (0-3.9ng/mL) and 5.5ng/mL (0-13.9ng/mL) were measured in patients with apparently refractory hypertension on 5mg ( $n=2$ ) and 10mg ( $n=5$ ) amlodipine respectively. A random plasma amlodipine concentration of 36.7ng/mL was measured in the patient described in the case study, who was receiving maximal doses of 10mg twice daily.

## CONCLUSION

An LC-MS-MS assay for amlodipine in human plasma was successfully developed and partially validated. The mean measured amlodipine concentrations in patients with uncontrolled hypertension were significantly lower than those observed in control patients. In some instances no amlodipine was detected, whilst in others concentrations were comparable to controls. In conclusion compliance testing may assist in the identification of poor adherence to drug therapy as a cause for refractory hypertension, allowing reallocation of resources to appropriate counselling. Where amlodipine is detected in the absence of concomitant improvement in blood pressure, drug concentration should be correlated with dose and the scope of investigation extended to include co-prescribed anti-hypertensive medication. Urine analysis may assist in identifying those patients who comply with their medication only prior to clinic. We acknowledge that other factors, such as genetics or drug interactions should also be considered.

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