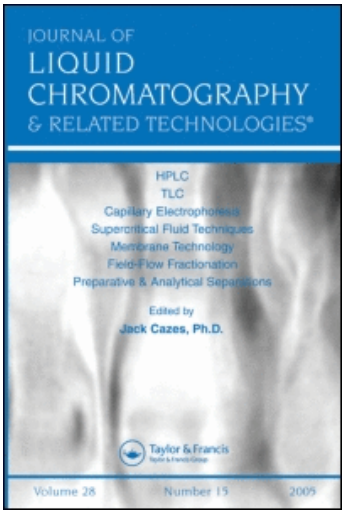


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# Modified Liquid Chromatographic Assay for Diltiazem and Metabolites in Human Plasma

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# MODIFIED LIQUID CHROMATOGRAPHIC ASSAY FOR DILTIAZEM AND METABOLITES IN HUMAN PLASMA

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## ABSTRACT

Diltiazem (DTZ) is an anti-hypertensive and anti-anginal agent which is also used clinically as a metabolic inhibitor to reduce cyclosporin-A metabolism. The present communication describes an HPLC/UV method for measuring DTZ and 3 of its major metabolites. This method has a LOQ below 2.5 μg/L and within run CV's ranging from 11.3 to 1.9% at concentrations of 2.5 and 100 μg/L, and between-run CV's ranging from 10.5 to 4.9% at concentrations of 75 and 350 μg/L, respectively. The method has been applied successfully to 11 renal transplant recipients taking a variety of other drugs.

## INTRODUCTION

Diltiazem (DTZ) is calcium channel blocking drug used pharmacologically in the treatment of hypertension and angina, and has also been used for an economic purpose by inhibiting the metabolism of the expensive immunosuppressant drug, cyclosporin-A (CsA).[1,2,3]

Diltiazem has been shown to be extensively metabolised to form 3 major metabolites N-monodesmethyl-diltiazem (MA), desacetyl-diltiazem (M1) and N-monodesmethyldesacetyl-diltiazem (M2). It has more than a 20-fold inter-subject variability in metabolite concentrations[4] and inter-species variability in its metabolic profile has been noted.[5] The primary route of DTZ metabolism is by the P450IIIA4 iso-enzyme system.[6] This isoenzyme is present in significant concentrations in the wall of the upper small intestine,[7,8,9,10] in addition to the liver, and has been demonstrated in animal studies to be a site of significant DTZ metabolism.[11,12] Its is perhaps not surprising, therefore, that DTZ suffers from a high "first pass" effect as the oral dose is absorbed from the gastric lumen.

There are also a number of commercially available DTZ formulations, including various "extended release" products. Whilst there are pharmacokinetic studies comparing two or three of these formulations,[13,14,15,16,17,18,19] there is very limited data in the full range of formulations and applications of DTZ, particularly metabolic interactions with other substrates for the P450IIIA4 isoenzyme, such as the clinically important "cyclosporin-A-sparing effect".[1,3,20,21,22,23]



Previous methods for analysis of DTZ (and its metabolites) have been reported, but may involve complicated equipment which may not be readily available in most laboratories,[24,25] or may have confined themselves to parent DTZ.[26,17] One early approach, which required minimal sample purification, appeared to limit column life and may have plasma interferences from patient samples, which could cause problems[27] or were less sensitive than the method described.[28,29]

The present method was developed from that kindly provided by LP Hackett and coworkers (personal communication, 1993), and describes a liquid chromatographic/UV method for the simultaneous determination of DTZ and the 3 metabolites in human plasma samples, applicable to pharmacokinetic studies.

## MATERIALS AND METHODS

### Stock Solutions

Separate stock solutions (100 mg base /L) of the pure substances, DTZ, MA (fumarate salt), M1 and M2 (kindly provided by Marion Merrell Dow Research Institute, Cincinnati, Ohio), were prepared in glass-distilled deionised water and these solutions serially diluted into single mixture of 10 mg/L and 1

mg/L of each compound. Desipramine HCl (Ciba-Geigy Australia Ltd, Sydney), was adopted as the internal standard and a 5 mg/L solution prepared. These solutions were held at -20°C between assays. All solvents were HPLC grade (BDH Laboratory Supplies, Poole, England) and phosphate buffer was Univar grade (Ajax Chemicals, Auburn, New South Wales, Australia).

## Plasma Extraction

Calibration standards were prepared in 1.0 mL of human plasma (previously shown to be devoid of interferences in the chromatographic system described) at concentrations of 10, 50 100, 250, 500 and 750 μg/L of DTZ and each of the DTZ-metabolites. Patient heparinised plasma samples (1.0 mL) and quality control samples, were aliquoted into 15 mL screw-capped extraction tubes in parallel with this calibration curve. Each tube was spiked with the internal standard (50 μL), basified with 100μL $Na_2HPO_4$ / $K_2HPO_4$ (50 mM, pH=7.5) and vortex-mixed briefly. Diethyl ether (5 mL) was added before capping and shaking horizontally for 20 min at 100 rpm. Phases were separated by centrifugation (10 min at 3000 rpm) followed by snap-freezing in a dry-ice/ethanol bath. The organic phase was decanted into a conical tube containing 100μL of 50 mM HCl. Tubes were vortex-mixed for a further 60 sec and the HCl phase snap-frozen and ether phase decanted to waste. The acid extract was transferred to auto-sampler tubes and 50 μL injected into the HPLC system described.



## Chromatography

The reverse phase chromatography was performed isocratically using a mobile phase comprising acetonitrile: $Na_2HPO_4$ (40mM, pH=5.5) (25:75) pumped at 1.0 mL/min (Spectra Physics, model P4000) through a 5μm reverse phase column (Lichrocart, RP-SelectB, 10 cm x 4 mm id, part #50829, E. Merck, Darmstadt, Germany), maintained at 40°C and the eluted substances detected by UV absorption at 215 nm (Spectra Physics, model AS2000). Aliquots (50μL) of extracted samples were injected using an autosampler (SpectraPhysics, model AS3000) and quantitation provided by a soft-ware data system (SpectraSystem version 1.2, Spectra Physics).

## Statistical Considerations and Patient Studies

Assay performance (precision and accuracy) was assessed by extracting replicates of plasma solutions containing DTZ and each of the 3 metabolites at 2.5, 10 and 100 mg/L within a single run (n = 6), and at 75 and 350 mg/L

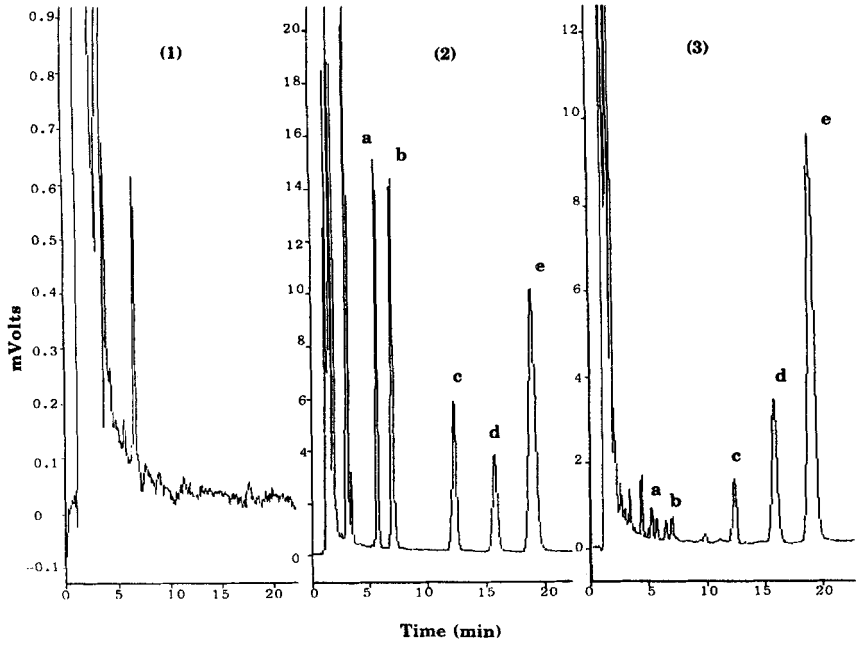


**Figure 1**. Sample chromatograms showing; panel (1) a "blank" plasma extracted from a patient not taking DTZ, panel (2) a plasma extracted from a sample spiked with 100 µg/L of M2 (a), M1 (b), MA (c), DTZ (d) and internal standard (e), and panel (3) a plasma sample from a renal transplant patient prescribed 60 mg DTZ tds. Note that the vertical attenuation varies in the 3 panels.



between runs (n=6). The robustness of the assay was assessed by application to samples from 11 unselected renal transplant recipients taking DTZ, plus a variety of other medications (including aspirin, atenolol, azathioprine, cephalexin, cyclosporin-A, frusemide, insulin, nifedipine, prazosin, prednisolone, ranitidine, sorbide nitrate), as this was the intended population for the initial application of the method. These patient samples included, were received by this laboratory for routine therapeutic drug monitoring assays of other drugs and, where DTZ was indicated, as part of "other drug therapy" for the patient on the assay request form.

## RESULTS

Examples of the 3 chromatograms obtained using the method described, are shown in Figure 1. This shows samples of; (a) a "blank" sample (drug-free

**Table 1**

**Precision and Accuracy Study Results for Parent Diltiazem (DTZ) and the 3 DTZ Metabolites***

| Compound | Concentration added (μg/L) | Measured Concentration (mean ± SD) (μg/L) | CV% |
|---|---|---|---|
| **Within run (n = 6):** | | | |
| DTZ | 2.5 | 3.36 ± 0.38 | 11.2 |
| | 10 | 9.67 ± 0.78 | 8.1 |
| | 100 | 91.8 ± 1.75 | 1.9 |
| MA | 2.5 | 3.46 ± 0.39 | 11.2 |
| | 10 | 10.34 ± 0.43 | 4.2 |
| | 100 | 91.9 ± 0.82 | 0.9 |
| M1 | 2.5 | 3.57 ± 0.33 | 9.1 |
| | 10 | 10.31 ± 0.50 | 4.9 |
| | 100 | 95.0 ± 2.97 | 3.1 |
| M2 | 2.5 | 3.76 ± 0.43 | 11.3 |
| | 10 | 10.41 ± 0.40 | 3.8 |
| | 100 | 91.6 ± 1.11 | 1.2 |
| **Between run (n = 6):** | | | |
| DTZ | 75 | 81.5 ± 0.40 | 4.9 |
| | 350 | 401.4 ± 41.2 | 10.3 |
| MA | 75 | 68.0 ± 7.2 | 10.5 |
| | 350 | 386.5 ± 28.8 | 7.5 |
| M1 | 75 | 72.6 ± 7.2 | 9.9 |
| | 350 | 389.9 ± 35.7 | 9.2 |
| M2 | 75 | 67.2 ± 5.7 | 8.4 |
| | 350 | 350.8 ± 30.8 | 8.8 |

* MA=N-monodesmethyl-DTZ, M1=desacetyl-DTZ, M2=N-monodesmethyl-desacetyl-DTZ.

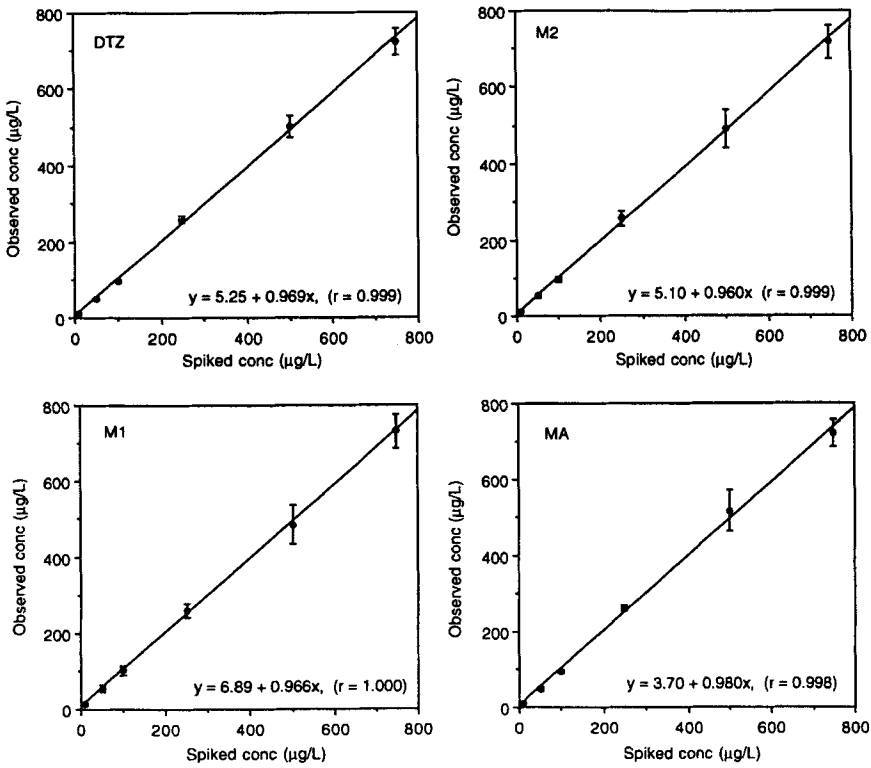


**Figure 2.** Calibration stability data for parent DTZ and each of the 3 metabolites from 6 analytical runs of the method described. Data shown are means ● sd's. The regression line and correlation coefficient are also presented.

plasma extracted), (b) a sample spiked with 100 µg/L of the 4 DTZ compounds (DTZ, MA, M1 and M2) and the internal standard, and (c) a sample from a patient taking 60 mg DTZ tds at steady-state, showing the presence of the parent DTZ and the 3 metabolites. The retention times for these 5 compounds were DLZ = 15.8 min, MA = 12.2 min, M1 = 6.9 min, M2 = 5.6 min and the internal standard = 18.9 min.

Whilst there were other peaks observed in some patient samples, presumably representing other medication (and/or metabolites) or endogenous substances, none were found to co-chromatograph with the 4 DTZ compounds of interest or internal standard.

The results of the precision and accuracy studies are presented in Table 1, which shows acceptable data both within and between runs. The CV at the lowest concentration studied (2.5 μg/L) was around 11%.

Figure 2 shows the stability of the calibration curves for DTZ and each of the 3 metabolites presented as means ± standard deviations (n=6). The regression line and correlation coefficient (r) is presented on the relevant figure for each compound.

## DISCUSSION

The assay of DTZ and its metabolites in a single assay has proved a challenge to many centres. The clear discrimination of 5 peaks (ie., 4 DTZ-related compounds plus the internal standard) in a single isocratic run from a plasma extract is seldom easy, particularly, where patient samples will contain other drugs and their metabolites, as well as, endogenous substances. Furthermore, the patients studied were from a renal transplant population where a spectrum of other drugs (including cyclosporin-A and metabolites) present would be expected. This patient population had biochemical indices of renal function at or above the upper limit of normal, which might result in an increased likelihood of interference from a variety of endogenous substances.

The concentration of 2.5 μg/L, which was adopted as the limit of quantification (LOQ) for practical purposes, could be viewed as conservative. One could go to lower concentrations if one applied the method acceptance criteria recommendations of adopting a 20% CV at the LOQ (precision and accuracy, within and between runs).[30]

Peak resolution was found to be sensitive to the pH of the mobile phase of the method described. A particular feature of DTZ and metabolites are their stability, as they have been reported to break-down on storage.[31] In the present study, it was found that storage of both stock aqueous solutions or patient samples at -20°C, was not associated with detectable deterioration over a period of at least 1 month, when compared with freshly prepared solutions.

The HPLC/UV method described for the assay of DTZ and 3 of its metabolites in human heparinised plasma, has acceptable precision and accuracy, as well as sensitivity and specificity suitable for clinical and/or pharmacokinetic applications. The sample preparation is no more complex than many other drug analyses and should not pose a significant problem for laboratories with experience in this field.

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