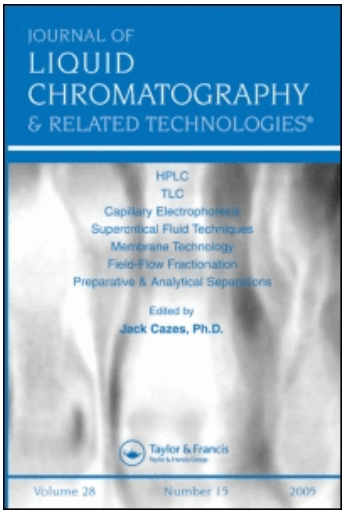

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HIGH THROUGHPUT ASSAY METHOD FOR THE QUANTITATION OF TRIMETAZIDINE IN HUMAN PLASMA BY LC/MS, WITH SELECTED REACTION MONITORING

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ABSTRACT

Following oral ingestion (40mg), trimetazidine was quantified in human plasma, using selected reaction monitoring (SRM). The simplicity of the liquid-liquid extraction procedure, together with the sensitivity and specificity afforded by LC-MS/MS, resulted in a highly sensitive and robust assay method in which 200-250 samples were processed per day.

Extraction from plasma into hexane/dichloromethane (1+1) and acetylation with acetic anhydride in the organic phase was followed by a novel back-extraction into water and in situ generated acetic acid. This acetylation procedure was found to be necessary to overcome significant carry-over problems encountered with the underivatised analyte. Separation was achieved on a Pheno-

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menex® Luna C_8 reversed phase column and detection by tandem mass spectrometry, with a sample turn-around time of 3 minutes.

INTRODUCTION

Trimetazidine, 1-[(2,3,4-trimetoxyphenyl)methyl] piperazine (Figure 1a), is a coronary vasodilator,[1] which has been used for many years worldwide in the treatment of ischaemic heart disease.[2]

Sensitive methods for the determination of trimetazidine in biological fluids have been described[3,4] and in this paper, a new method is described for the quantitation of trimetazidine in human plasma, following acetylation (Figure 1b). The main benefit of this new procedure over previously published methods[3,4] is the less laborious sample preparation procedure, as well as improved specificity. Although a qualitative LC/MS method for the identification of trimetazidine and its metabolites in urine has been published,[2] no high throughput LC-MS/MS assay method has appeared in the literature for the quantitation of trimetazidine in plasma. Bromet and Courte[4] describe an HPLC assay of trimetazidine with fluorometric detection, following dansylation..

A GC/MS method for the determination of trimetazidine in a number of biological fluids, following *tert.*-butyldimethylsilation, was reported by Fay *et al.*[3] Using 1 mL plasma, a limit of quantification (LOQ) of 1 ng/mL in plasma was obtained, at a signal-to-noise ratio of 3:1. The sensitivity of the assay method described in this publication compares favourably, as a LOQ of 1.51 ng/mL was obtained, at a signal-to-noise ratio of 20:1, using 0.5 mL plasma.

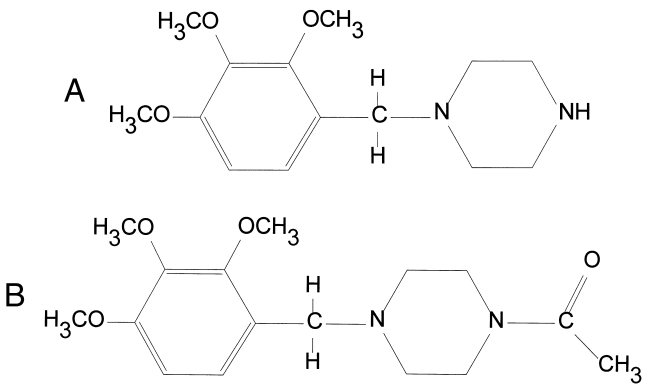

Figure 1. (a) 1-[(2,3,4-trimethoxyphenyl)methyl] piperazine. (b) 1-(2,3,4-trimetoxybenzyl)-4-(N)-acetylpiperazine.

EXPERIMENTAL

Reagents and Materials

Trimetazidine dihydrochloride was obtained from Cipla (Bombay, India). HPLC-grade acetonitrile, methanol, hexane, and dichloromethane (B & J brand™) were purchased from Baxter (Muskegon, MI, USA). Sodium hydroxide (Fluka, Buchs, Switzerland), as well as acetic anhydride and acetic acid (Merck, Darmstadt, Germany) were used without further purification. All water used was purified by RO 20SA reverse osmosis and Milli-Q® polishing system (Millipore, Bedford, MA, USA).

Extraction Procedure

To 0.5 mL plasma in 5 mL glass ampoules were added 0.2 mL NaOH (2M) and 3 mL hexane : dichloromethane (1 : 1). The ampoules were vortexed (1.5 min) and centrifuged in a Megafuge 1.0R for 2 min (Heraeus, Hanau, Germany), at 1300*G*. The aqueous layer was frozen on a Fryka Polar KP 250 cooling plate (Kältetechnik, Esslingen, Germany), at ca. -30°C and the organic layer decanted into another 5 mL ampoule. Acetic anhydride was added (10 μL), the sample briefly vortexed, and the solution allowed to stand at room temperature for 20 min.

Water (0.2 mL) was added and the sample vortexed for 1.5 min causing basic analytes to be back-extracted into the *in situ* generated acetic acid. After centrifugation (2 min, 1300*G*), the aqueous layer was frozen and the organic layer discarded. The aqueous layer was thawed, briefly placed under a stream of nitrogen in order to remove any residual organic solvent, and 10 μL injected onto the HPLC column.

Calibration

A series of trimetazidine spiking solutions was prepared by dissolving trimetazidine in methanol, and calibration standards and quality controls were prepared by spiking individual pools of blank plasma to known concentrations. A concentration range spanning 383 – 1.51 ng/mL was prepared in plasma and used to validate the assay method. Sufficient calibration standards and quality controls were prepared to validate the assay method and serve as calibrators and quality controls during the assaying of the study sample batches.

Instrumental Conditions

Automated instrumental optimisation of the transition from the molecular ion of N-acetylated trimetazidine (*m/z* 309.0) to the predominant daughter ion (*m/z* 181.0), was performed on a P-E Sciex API 2000 LC/MS instrument interfaced to a computer using MassChrom 1.1 software (Perkin-Elmer, Foster City, CA, USA). The nebuliser, turbo, and curtain gases were set at 50, 80, and 55 units, respectively, with the turbo gas temperature fixed at 400°C. The electrospray needle was maintained at 400°C, and at a potential of 5000V. The mass dependent parameters RO2, RO3, IQ3, and IQ2 were –22, –27, –46, and –17 V, respectively.

Separation was achieved on a Phenomenex ® Luna C_8 (2) 5 µ (150 × 2 mm) stainless steel column (Phenomenex, Torrence, CA, USA). The mobile phase consisted of methanol : acetonitrile : water : glacial acetic acid (180 + 20 + 800 + 5, v/v) and was delivered at 0.3 mL/min by a Hewlett-Packard series 1050 pump (Hewlett-Packard, Palo, Alto, CA, USA). 10 µL of the final extract was injected onto the column at ambient temperatures by a Hewlett-Packard series 1100 autosampler, and the retention time of the acetylated analyte was approximately 1.75 min. Sample extracts resided in autosampler vials containing small volume glass inserts and were kept at 5°C while awaiting sample injection.

Analyte Stability in Matrix

Trimetazidine plasma standards at high, medium, and low concentrations, which had been prepared three months previously and stored at -20°C, were extracted together with freshly prepared plasma standards of the same concentration, each in triplicate. A mean difference in response of 2.7% between freshly prepared and stored plasma standards was deemed negligible, and it was concluded that trimetazidine is stable in human plasma at -20°C for at least three months.

On-Instrument Stability

The response of a single extracted sample, which was repeatedly injected, was monitored over five hours during which the sample was on the instrument. The analyte peak area showed no reduction and trimetazidine can be considered stable in the final reconstituted solution for at least five hours.

Extraction Efficiency

The mean efficiency of the extraction procedure, determined in triplicate at high, medium, and low concentrations was found to be 75%.

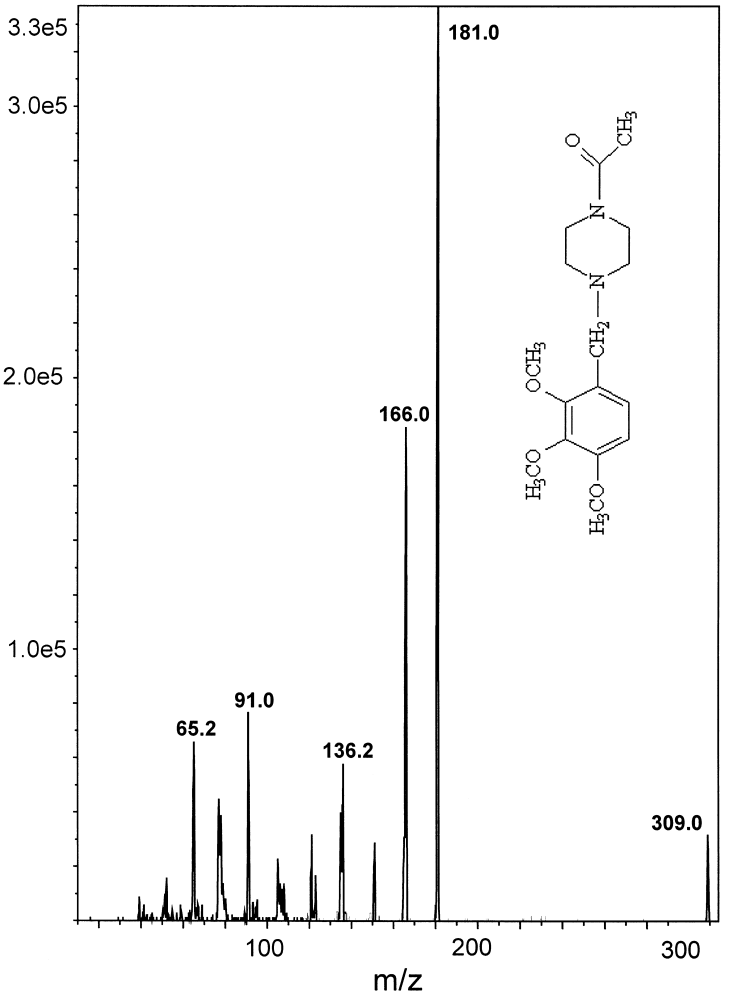

Figure 2. Spectrum of *N*-acetylated trimetazedine showing the M^{+1} (m/z 309.0) ion isolated, and predominant daughter (m/z 181.0) monitored in the MRM procedure.

Chromatography

Good chromatographic results were obtained for the *N*-acetylated analyte (Fig. 3), with a retention time of ~ 1.75 min.

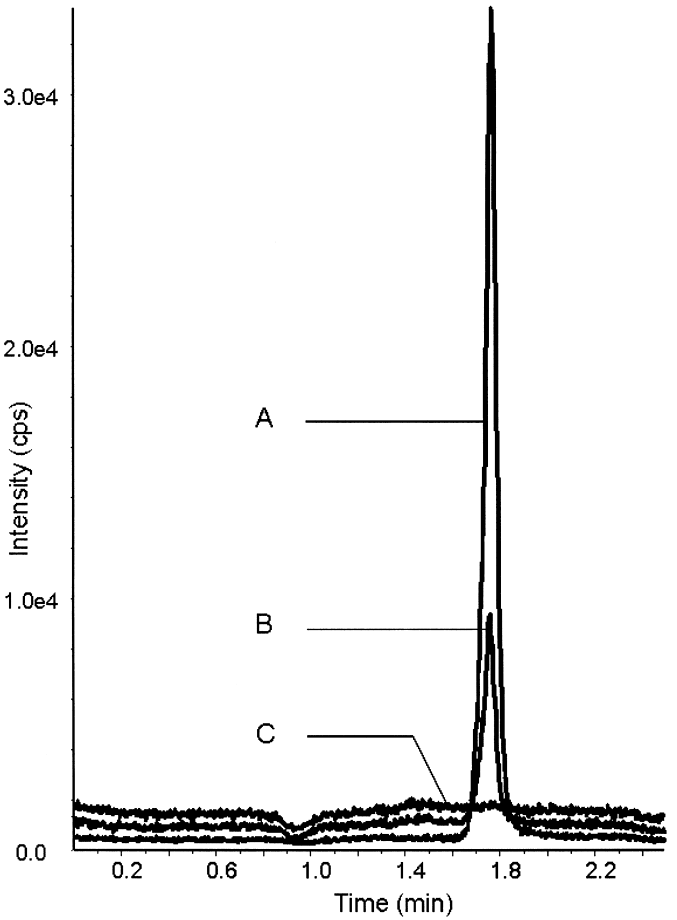

Figure 3. Overlaid chromatograms of (A) a study plasma sample 24h after a 40mg oral dose of trimetzidine dihydrochloride, (B) a 1.51 ng/mL plasma calibration standard and (C) a blank plasma extract.

Sensitivity

The lowest calibration standard that was found to perform consistently with respect to accuracy and precision (CV% < 20) was 1.51 ng/mL, at a mean signal-to-noise ratio of 20:1 when using 0.5 mL plasma, and injecting 10 µL onto the HPLC column.

Matrix Effects

Matuszewski *et al*.[5] have reported that undetected endogenous matrix components, which co-elute with the compound of interest, may adversely affect both the extent and reproducibility of analyte ionisation. In order to determine the extent of these so-called matrix effects, blank plasma from six different sources was extracted and then spiked to a known analyte concentration. Following injection, the peak areas were compared, and the reproducibility interpreted as an indication of the presence or absence of matrix effects. The data observed indicated that there were no matrix effects at the tested concentration (317 ng/mL), as the CV% (4.5%, n = 6) did not differ from the CV% reflected in the intra- and inter-day accuracy and precision (Tables 1 and 2).

Method Validation

Nine calibration standards, which spanned a concentration range between 1.51 and 383 ng/mL, were extracted and used to construct a calibration line. A range of quality controls, which had been independently prepared and which spanned approximately the same concentration range, were extracted ($n = 5$) and used to validate the assay method. The performance of the assay method with respect to accuracy and precision is presented in Tables 1 and 2. The inter-day data were obtained with quality controls interspersed in each batch of actual clinical study samples assayed on the dates indicated in Table 2. We consider this to be a more accurate reflection of the assay method's inter-day performance than the data normally presented in assay method publications; (quality controls assayed in single batches in five-fold on three consecutive days).

Table 1. Intra-Day Accuracy and Precision of Quality Controls

Nominal Concentration (ng/mL)	Measured Quality Control Concentration (ng/mL)	n	CV %
346	350	5	4.5
173	177	5	2.5
86.4	91.9	5	3.1
43.2	46.3	5	6.4
21.6	22.4	5	4.0
10.8	10.9	5	4.7
5.39	5.46	5	5.9
2.68	2.70	5	8.9

Table 2. Inter-Day Accuracy and Precision of Quality Controls

Nominal Concentration (ng/mL)	Date	Measured Quality Control Concentration (ng/mL)	CV %
96.2	25/05	88.7	
	26/05	104	
	27/05	93.5	
	28/05	91.5	
	29/05	99.9	
	Mean	95.6	6.8
43.2	25/05	41.1	
		40.1	
	26/05	41.2	
		39.9	
	27/05	43.7	
		42.6	
	28/05	38.8	
		41.7	
	29/05	42.3	
		44.6	
	Mean	41.2	4.3
21.6	25/05	22.7	
		21.4	
	26/05	23.3	
		20.0	
	27/05	24.5	
		21.5	
	28/05	20.3	
		21.5	
	29/05	19.2	
		19.7	
	Mean	21.4	7.9
10.8	25/05	11.1	
		10.1	
	26/05	11.0	
		9.96	
	27/05	10.2	
		11.1	
	28/05	10.0	
		9.53	
	29/05	9.97	
		9.37	
	Mean	10.2	6.1

Table 2. Continued

Nominal Concentration (ng/mL)	Date	Measured Quality Control Concentration (ng/mL)	CV %
2.68	25/05	3.10	
		2.44	
	26/05	2.85	
		2.42	
	27/05	2.07	
		2.61	
	28/05	2.54	
		2.48	
	29/05	2.63	
		2.69	
	Mean	2.58	10.6

Batch Processing

Enough calibration standards and quality controls were prepared to assay all subsequent assay batches and kept frozen at -20°C for this purpose. With each assay batch, these calibration standards and quality controls were thawed, extracted, and dispersed throughout the batch.

RESULTS AND DISCUSSION

Method Optimisation

Initial attempts at developing a LC-MS/MS assay method for the quantitation of underivatized trimetazidine failed due to carry-over proving to be a significant problem. An interesting feature was the fact that there was carry-over of trimetazidine only and absolutely none of doxepin, the internal standard used at that stage. No discernible pattern of carry-over with respect to a percentage of the previous injection could be observed. Known sources of carry-over were investigated, sequentially, in an attempt to pinpoint the cause.

The first approach, was to include a needle rinsing sequence in the autosampling method, which proved fruitless. Following this, the needle seat, loop, rheodyne valve, and connecting tubing were all checked, rinsed, and PEEK tubing substituted with stainless steel, resulting in no improvement. The analytical column was substituted by an identical new column, which improved, but did

not solve the problem, and even a number of injections made without a column in-line, did not totally eliminate the carry-over. Furthermore, the baselines became noisy, and it appeared that there was a gradual bleed of analyte into the detector. Although we were unable to find a satisfactory explanation for this phenomenon, it was concluded that there was a gradual systemic build-up of trimetazidine (possibly adsorption on all the components between the injector and the ESI needle), and that the acidic injection plug eluted differing amounts of residual trimetazidine from these components with each successive injection.

Another analytical obstacle was the poor peak shape. Secondary amines, such as trimetazidine, are often associated with peak tailing under reverse-phase conditions, and the limitations that LC/MS-ESI places on LC mobile phases greatly diminishes the 'arsenal' available to improve chromatography. Attempts at changing the nature and proportion of the organic component in the mobile phase, as well as the use of various organic acids at several concentrations, did not improve the chromatography appreciably. The use of a C_{18} as opposed to a C_8 analytical column also proved unsuccessful.

A tendency of trimetazidine to form multiple peaks, probably due to pH perturbations in the eluting mobile phase caused by the injection solvent, was also not conducive to trouble-free chromatography. Although this effect was investigated in various mobile phases, with the extent of the effect either waxing or waning with each new composition, we were unable to eliminate this phenomenon altogether.

The poor performance of the assay method as a whole at that stage, prompted us to consider derivatising the analyte, although this would complicate the assay procedure somewhat. The first and only derivatisation reaction attempted was the acetylation procedure described. To minimise sample preparation time, we reasoned that the excess acetic anhydride that remained in the organic solvent after derivatisation would be rapidly hydrolysed to acetic acid were it to come into contact with water, and that this situation could conveniently be used to back-extract acetylated trimetazidine into water, provided that the water would be rendered acidic enough by the *in situ* generated acetic acid. This was in fact shown to be the case.

This assay method proved to be superior to the initial procedure. Not only was there no carry-over of acetylated trimetazidine, and the peak symmetrical, but no peak multiplicity was observed either.

Internal Standard

While it is common practice to use an internal standard, especially in assays in which the extraction procedure involves several steps, its addition does introduce another variable into the assay procedure. Therefore, unless the inter-

nal standard is eminently suitable, it may actually increase the variability of the assay method instead of reducing it. In the case of LC/MS, the ideal internal standard does exist in the form of the isotope-labelled analyte, but unfortunately the availability of such an internal standard is rather limited and often depends on custom synthesis which is not always simple but always costly. In the absence of such an ideal internal standard, we tried out several compounds as internal standards but found none that improved the variability of the assay method; hence, the decision to validate the assay without the use of an internal standard.

Since we are aware that constant ionisation is not always readily achieved when using API-MS/MS, especially during the assay of large batches, the calibration standards and controls are not grouped somewhere in the batch, but are always dispersed evenly over the entire batch. Unknown samples, in groups of eight, were assayed between calibration standards, quality controls or both. The fact that at least every ninth sample was a control sample, gave the authors confidence that the data generated from the unknown samples were reliable. The inter-day quality control results obtained during the assaying of several hundred clinical study samples and presented in Table 2, attest to the good performance of this assay procedure under actual clinical study conditions.

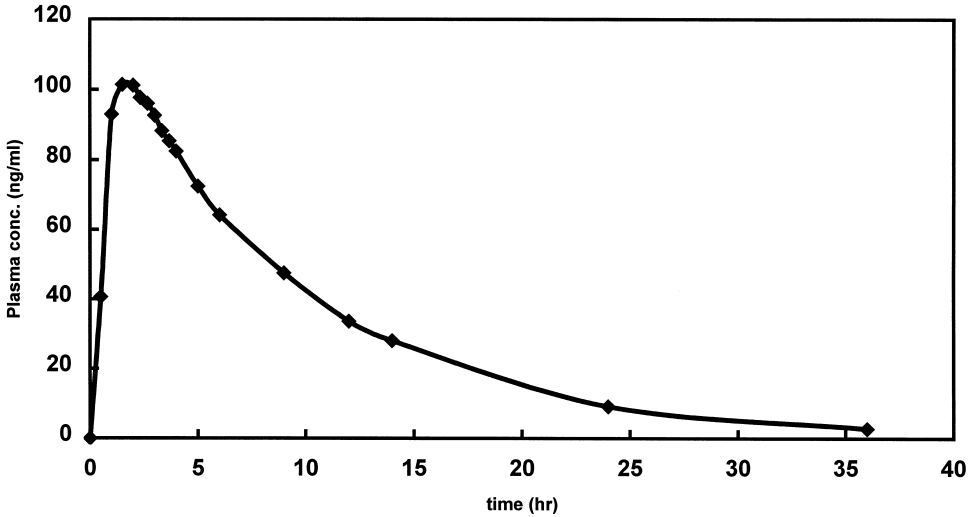

Figure 4. Mean plasma profile following a single 40 mg oral dose of trimetazidine.

Table 3. Mean Calculated Pharmacokinetic Parameters (n = 24)

C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC(0-t_{last}) (ng/mL*h)	AUC(0-∞) (ng/mL*h)
Mean: 112	Mean: 2.13	Mean: 6.27	Mean: 1080	Mean: 1107
Min: 80.0	Min: 1.00	Min: 5.00	Min: 596	Min: 621
Max: 192	Max: 3.67	Max: 7.93	Max: 1510	Max: 1540
CV% 21.3	CV% 47.2	CV% 13.0	CV% 22.1	CV% 22.2

Application

This assay method was employed in a pharmacokinetic study carried out with human volunteers following a single oral dose of trimetazidine. A mean plasma concentration versus time profile is presented in Figure 4 and relevant pharmacokinetic parameters in Table 3. The assay method was found to be robust and rapid; tandem mass spectrometry ensured that the assay method was highly specific and sensitive, and 200 - 250 samples were processed per day. The acetylation procedure, followed by back-extraction into *in situ* generated acetic acid, could possibly serve as a model procedure applicable to many primary and secondary amine compounds exhibiting the problems, which we initially encountered with trimetazidine.

REFERENCES

1. Elks, J.; Gannelin, C.R.*Dictionary of Drug*; Chapman and Hall: London, New York, 2990.
2. Jackson, P.J.; Brownsill, R.D.; Taylor, A.R.; Resplandy, G.; Walther, B.; Schwietert, H.R. Xenobiotica **1996**, *26* (2), 221-228.
3. Fay, L.; Michael, G.; Goupit, P.; Harpey, C.; Prost, M. J. Chromatogr. **1989**, *490*, 198-205.
4. Courte, S.; Bromet, N. J. Chromatogr. **1981**, *224*, 162-167.
5. Matuszewski, B.K.; Constanzer, M.L.; Chavez-Eng, C.M. Anal Chem. **1998**, *70*, 882-889.

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