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Note

Analysis of a novel cardiac stimulant, UK-61 260, in human plasma by gas chromatography–mass spectrometry with selected-ion monitoring

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6-(2,4-Dimethyl-1H-imidazol-1-yl)-8-methyl-2(1H)-quinolinone (UK-61 260, I) is a novel, orally active, non-glycosidic, cardiac stimulant with additional vasodilator properties [1]. Currently I is undergoing extensive clinical evaluation for the treatment of congestive heart failure.

A high-performance liquid chromatographic assay using ultraviolet detection has been used for pharmacokinetic studies in the dog [2] and in early Phase 1 studies in man [3]. However, this method with a detection limit of 3 ng/ml is insufficiently sensitive for widespread use in human studies. This paper describes a sensitive method for the determination of I in human plasma involving solid-phase extraction (SPE) followed by derivatisation and capillary gas chromatography (GC) with selected-ion monitoring (SIM); the method has a limit of detection of 0.2 ng/ml. Modifications to allow the method to be used to determine I in human urine are also described.

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EXPERIMENTAL

Chemicals and reagents

All chemicals and reagents used for the assay were of HPLC-reagent grade, unless otherwise stated. I methane sulphonate salt dihydrate (Fig. 1) and the internal standard (UK-63 127, free base) were prepared in the Medicinal Chemistry Department at Pfizer Central Research. Solid-phase extraction cartridges (SPE C_{18}) were purchased from J.T. Baker (Deventer, The Netherlands). Water was deionized and freshly glass-distilled before use. The silylating reagent, N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide, containing 1% *tert.*-butyldimethylchlorosilane (MTBSTFA) was purchased from Regis Chemicals (Morton Grove, IL, U.S.A.). Since interference at the retention time of the I derivative had been seen in certain batches of MTBSTFA, batches of reagent were tested for interference before use in the analytical procedure.

Preparation of solutions

Stock solutions of I and internal standard were prepared in water and methanol, respectively, at a concentration of 100 μg/ml equivalents of base and stored at 4 °C in the dark. Stock solutions, under these conditions, were stable for six months. These solutions were diluted before use with methanol to a concentration of 1 μg/ml. These solutions were prepared monthly and stored at 4 °C. The solution of I was further diluted on a daily basis to 0.1 μg/ml with methanol. Phosphate buffer was prepared by mixing solutions of sodium dihydrogenphosphate monohydrate (1.38 g/l) and disodium hydrogenphosphate dihydrate (1.78 g/l) in appropriate amounts to give a final pH of 7.5.

Pre-treatment of SPE cartridges

SPE C_{18} cartridges were batch-tested for recovery of I by adding I to blank plasma at a concentration of 2.0 ng/ml and taking the samples ($n=6$) through the extraction procedure. Internal standard was added to the methanol eluate

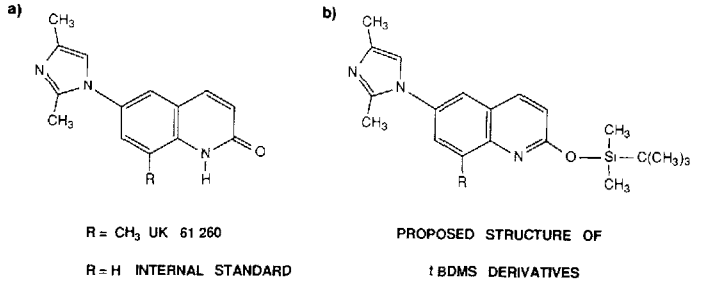

Fig. 1. (a) Structure of I (UK 61 260) and internal standard. (b) Proposed structure of *tert.*-butyldimethylsilyl (tBDMS) derivatives.

from the SPE cartridges and the samples were taken through the derivatisation procedure. The mean peak-height ratio was compared with that obtained from directly injected standards and batches giving less than a mean recovery of 85% were rejected.

The cartridges were pre-treated before use by washing sequentially with 3×1 ml methanol, 2×1 ml water and 1 ml phosphate buffer.

Extraction procedure

Plasma was thawed, vortex-mixed briefly and centrifuged before use. Plasma (1.0 ml), after the addition of 5 μl of internal standard solution, was mixed with phosphate buffer (1.0 ml), using a vortex mixer. The buffered plasma was applied to a pre-treated SPE C_{18} cartridge and the analytes were absorbed by applying a light vacuum. Residual buffered plasma in the sample tubes was washed onto the cartridge with 2×1 ml water. Methanol–water (1:1, v/v, 0.2 ml) was applied to the cartridge and the eluate discarded. The analytes were eluted from the cartridge with 2×0.3 ml methanol and collected into a clean glass tube. The methanol was removed at 50°C under a gentle stream of nitrogen. The residue was reconstituted by vortexing with 25 μl methyl *tert.*-butyl ether and 25 μl of MTBSTFA. The air in the tube was replaced with nitrogen and the tubes were tightly capped. The tubes were allowed to stand at room temperature for 1 h in order to derivatise the analytes. The contents of the tube were then transferred to a 100-μl conical auto-injector vial. Injection volumes of 2 μl were used for gas chromatography–mass spectrometry (GC–MS).

Instrumentation

GC–MS was performed on a Hewlett-Packard HP5985A system operated in the electron-impact (EI) mode. The gas chromatograph was equipped with a fused-silica capillary column (CP-Sil 8CB, 25 m×0.32 mm I.D., 0.4 μm film thickness, Chrompack, Middelburg, The Netherlands). The column was directly coupled to the ion source. Helium was used as the carrier gas at a head pressure of 50kPa. Total flow-rate, including septum purge and vent, was 50 ml/min. Automatic splitless injection was accomplished using a Hewlett-Packard HP7672A injector with a splitless insert packed with untreated glass wool maintained at 300°C. The complete insert was deactivated by immersing in a 2% (w/v) solution of Carbowax 20M in chloroform for at least 2 h. This insert was replaced daily. The column temperature was programmed to increase from 200 to 300°C at a rate of 20°C/min. The interface and source temperatures were 275 and 200°C, respectively. The electron energy was 70 eV. Data acquisition was performed in the SIM mode at *m/z* 296 and 310 for the internal standard and I derivatives, respectively.

Storage of samples

Plasma and urine samples from pharmacokinetic studies were transported frozen to BCO Analytical Services (Breda, The Netherlands) packed in dry ice and subsequently kept at −20 °C until analysis.

Calibration curves

Calibration standards were prepared by adding known amounts of I to control human plasma. Volumes of 2, 4, 8 and 16 μl (from 1 μg/ml stock solution) and 2, 5 and 10 μl (from 0.1 μg/ml stock solution) were added to 1 ml plasma and processed as described above. A calibration line covering the range 0.2–16 ng/ml using seven points was prepared for each daily assay run. A control plasma sample without I was also included to check for background interference at the retention time of the derivative. For studies at doses above 2 mg an extra point at 20 ng/ml was included in the calibration curve.

Analysis of results

The peak-height ratio of *m/z* 310/296 (I derivative/internal standard derivative) was calculated for all samples using the automatic routines of the Hewlett-Packard data system. The calibration line was calculated by unweighted linear regression of peak-height ratio on concentration of calibration samples. The concentration of I in test samples was calculated using the regression parameters.

RESULTS AND DISCUSSION

Underivatised I showed absorption and tailing when subjected to GC, probably due to the polar nature of the cyclic amide function. Quinoxalinones, a related compound class, have been shown to react with most conventional alkylating agents (e.g. N,N-dimethylformamide dimethyl acetal, diazomethane, methyl sulphate) but tend to form a mixture of the amide and phenolic tautomers [4]. However, quinoxalinones have been trimethylsilylated [4] producing a single component which was shown by NMR and IR spectroscopy to be the O-trimethylsilyl (O-TMS) ether. The O-TMS derivative of I was prepared in a similar fashion but was found to readily hydrolyse and show extensive mass spectral fragmentation. In contrast, the *tert.*-butyldimethylsilyl (tBDMS) derivative of I was readily formed by reaction with MTBSTFA and did not hydrolyse, even when the reagent was removed. By analogy with the quinoxalinones it is proposed that only the O-tBDMS derivative of I is formed since a single component is seen on GC. This derivative did not yield a molecular ion but showed a single fragment ion (*m/z* 310) in the mass spectrum which would correspond to a loss of the *tert.*-butyl group from the molecular ion (i.e. M−57). The derivative formed by the internal standard showed similar properties; viz. a single chromatographic peak and a single fragment ion

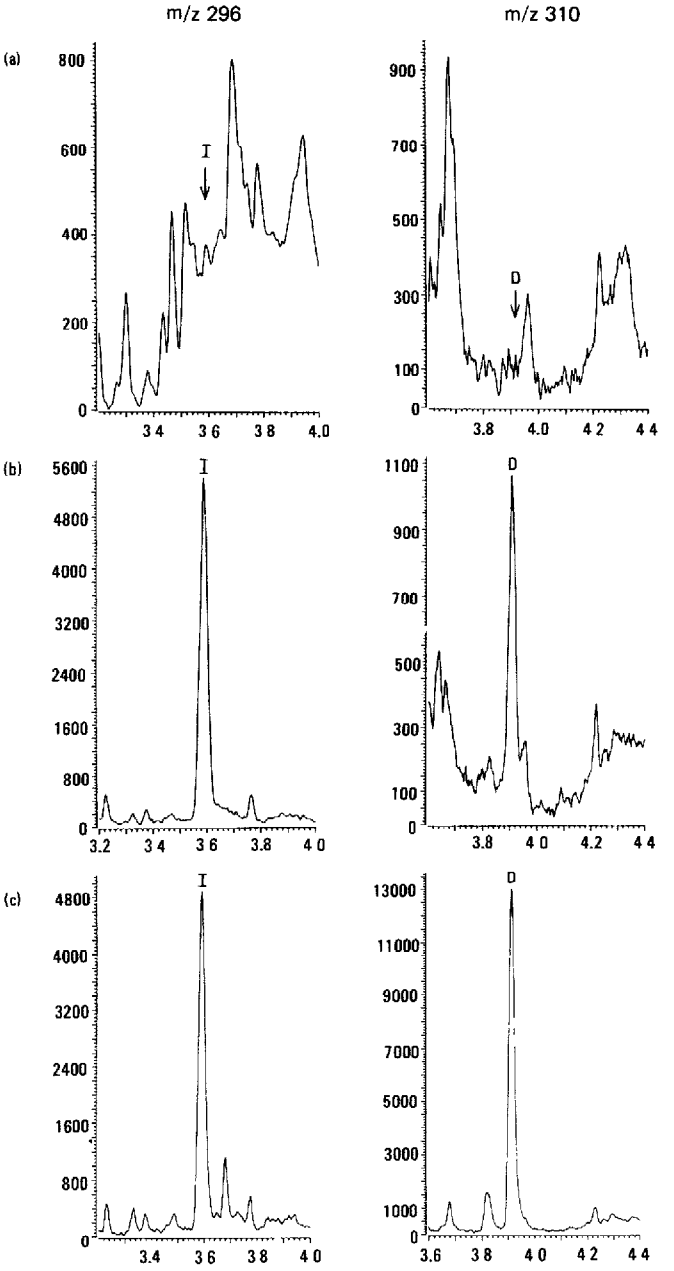

Fig. 2. Typical GC–SIM profiles at *m/z* 296 (internal standard) and *m/z* 310 showing (a) drug-free control plasma extract, (b) control plasma extract containing 0.5 ng/ml I and 5 ng/ml internal standard and (c) plasma extract of a human subject 2 h after oral administration of 1 mg I. Peaks: D = derivatised I (retention time 3.9 min); I = derivatised internal standard (retention time 3.6 min). All traces normalised.

in the mass spectrum at m/z 296 (M−57). These characteristics are highly suitable for a quantitative SIM assay since the major proportion of the ion current is contained in one ion.

The initial extraction method used in this assay had been a low-throughput, multi-stage liquid–liquid extraction procedure [5]. This was coupled to a manual GC–MS end-point using a dropping needle injector which also had a low sample throughput. The advantages with this original method were that the final derivatised extract was cleaner than the eventual SPE method and a larger proportion of the final extract could be injected. The cleaner extract obtained by the liquid–liquid extraction procedure meant less maintenance of the injector was required and a better signal-to-noise ratio at low concentration was achieved. For these reasons the liquid–liquid method had a lower limit of detection (<0.1 ng/ml) than the solid-phase method due to cleaner SIM profiles, but in practice the limit of detection of 0.2 ng/ml obtained with the solid-phase method is adequate for the clinical samples. The extraction method detailed was developed to enable large batches of samples to be processed with the minimum sample preparation. In conjunction with the autosampler the SPE assay met its goal of high throughput whilst retaining high sensitivity.

Fig. 2 shows typical SIM traces for the internal standard and I derivatives of a control plasma extract to which 0.5 ng/ml I had been added and an extract of plasma from a human subject dosed with I and found to contain 6.5 ng/ml I. Internal standard had been added to each sample at a concentration of 5 ng/ml. The figure also shows a typical SIM trace of a drug-free control plasma extract. The retention times of the internal standard and I derivatives were 3.6 and 3.9 min, respectively. The SIM chromatogram obtained from analysis of control human plasma shows no interference at the retention time of I.

Intra-assay variation

The within-day variation was investigated by processing control plasma samples containing known amounts of I. The mean variation of the analytical procedure was determined at 0.2, 0.4 and 2 ng/ml (Table I).

TABLE I

INTRA-ASSAY VARIATION FOR I IN HUMAN PLASMA

Concentration added (ng/ml)	Peak-height ratio m/z 310/296 (mean ± S.D.)	Coefficient of variation (%)	n
0.2	0.0571 ± 0.00469	8.2	8
0.4	0.0895 ± 0.00626	7.0	9
2.0	0.4971 ± 0.0277	5.6	10

TABLE II

INTER-ASSAY VARIATION OVER A FIVE-DAY PERIOD

Replicate analysis of a sample containing 1.06 ng/ml I.

Day	Found concentration (ng/ml)	Bias (%)
1	1.07	+0.9
2	1.05	−0.9
3	1.08	+1.9
4	1.03	−2.8
5	1.04	−1.9
Mean	1.05	1.7[a]

[a]Mean bias, value quoted is irrespective of sign.

TABLE III

ANALYSIS OF CONTROL PLASMA CONTAINING I AT CONCENTRATIONS UNKNOWN TO THE ANALYST

Concentration added (ng/ml)	Concentrations found ($n=2$) (ng/ml)	
0.92	0.91	0.92
1.63	1.54	1.59
0.41	0.40	0.55
1.22	1.16	1.10
0.20	0.20	0.20
1 93	2.03	2.09
0.61	0.53	0.54
10.2	10.8	10.5
6.10	6.19	5.73
16.3	16.7	16.5

Inter-assay variation

The inter-assay variation was determined by replicate analysis of quality control samples over a five-day period. These samples were 1-ml aliquots drawn from a larger sample prepared by adding I to a bulk volume of control human plasma at a concentration of 1.06 ng/ml (Table II). The inter-assay variation was also continuously monitored by re-analysis of patient and volunteer plasma samples. The mean difference for a group of eighty samples between the second assay compared with the first, regardless of sign, was 9%.

Accuracy

The concentration of I in control plasma samples to which drug had been added at concentrations unknown to the analyst was determined. Table III shows the result of ten samples assayed on two separate occasions.

Analysis of urine

The drug concentration in urine from subjects dosed with I was generally higher than the normal concentration range for plasma. Urine diluted with water or control urine was found to give low and irregular recoveries when taken through the described extraction procedure. It is believed that I and the internal standard are retained on the SPE matrix not only by non-polar interactions with the C_{18} groups but partly by ionic interactions of the basic moiety with uncapped silanol groups. The high ionic strength of urine may cause displacement of the analytes from these ionic binding sites. The large variation in ionic strength of urine could therefore cause the observed variable recovery due to non-retention of I and the internal standard. For this reason, control plasma was used as a matrix modifier to dilute and buffer the urine before analysis. It was necessary to dilute urine with a minumum five-fold excess of plasma to achieve consistant recoveries. Using this modification the limit of detection of I in urine is 0.5–2.0 ng/ml depending on the dilution factor. The batch-to-batch variability of recovery from plasma seen with SPE C_{18} cartridges may also be explained by the above binding criteria. It has been reported that non-uniform surface coverage of the silica with octadecasilyl groups or variation in the percentage carbon content of C_{18} bonded porous silicas can occur [6] which could affect the proportion of silanol sites available for ionic binding. Hence, any variation in the production of SPE C_{18} cartridges may result in variable recoveries of analytes containing a basic moiety.

Clinical samples

The procedure described has been used extensively for assay of plasma and urine samples from clinical studies in human volunteers and patients. The assay has been used for the analysis of plasma samples from drug interaction

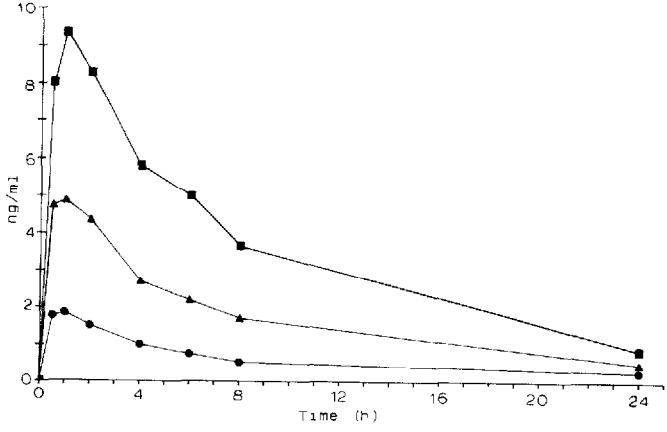

Fig. 3. Typical profiles for the plasma concentrations of I in man. Data from human subjects given single oral doses of 0.25 mg (●), 0.5 mg (▲) and 1.0 mg (■).

studies and proved suitable for use when I was co-administered with digoxin and frusemide. Fig. 3 shows typical plasma concentration–time profiles for I in three subjects from an open multicentre dose-ranging study of single oral doses of I in patients with chronic heart failure. The three subjects were given single oral doses of 0.25, 0.5 and 1.0 mg. Peak concentration in plasma was obtained after 1 h with an elimination half-life of 4 h.

CONCLUSION

To date, we have analysed over 2000 human plasma samples from different pharmacokinetic studies in both volunteers and patients. The assay has been in continuous use for a six-month period and has shown to be robust and reliable. A well trained laboratory technician is capable of analysing over 50 samples in a day and routinely obtain detection limits of less than 0.2 ng/ml.

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