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Short Communication

Liquid–liquid extraction and high-performance liquid chromatography for the determination of a novel antidysrhythmic agent (UK-68,798) in human urine

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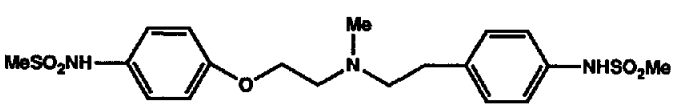
ABSTRACT

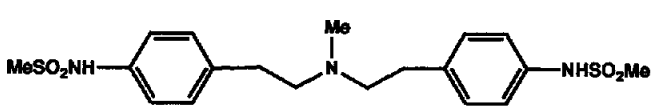
A routine method for the determination of a novel class III antidysrhythmic agent, 1-(4-methanesulphonamidophenoxy)-2-[N-(4-methanesulponamidophenethyl)-N-methylamino]ethane, in human urine has been developed. The method involves solvent extraction followed by high-performance liquid chromatography on an unmodified silica column with ultraviolet detection. Despite a low recovery of drug through the three-stage extraction procedure a reliable assay with high precision (coefficient of variation less than 6%) and a limit of determination of 2.5 ng/ml was achieved. The method has been applied to the analysis of samples following single oral and intravenous doses of 1–12.5 μg/kg of the drug to human volunteers.

INTRODUCTION

1-(4-Methanesulphonamidophenoxy)-2-[N-(4-methanesulphonamidophenethyl)-N-methylamino]ethane (I, UK-68,798, Fig. 1) is a novel class III antidysrhythmic agent [1,2] currently undergoing clinical evaluation.



UK-68,798



UK-69,308

Fig. 1. Structures of I (UK-68,798) and internal standard (UK-69,308).

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A radioimmunoassay procedure for the determination of I in human plasma has previously been described with a limit of determination of 50 pg/ml [3]. This sensitivity was required due to the low doses administered clinically. Due to a high proportion (about 60%) of the clearance of I being by the renal route an assay was required for routine analysis of the drug in urine. The higher concentrations of drug present in the urine, compared to plasma, facilitated the development of a high-performance liquid chromatographic (HPLC) assay.

This paper describes a liquid–liquid extraction procedure for isolation of I from urine and subsequent analysis by HPLC on an unmodified silica column with ultraviolet (UV) detection. The limit of quantitation of the method is 2.5 ng/ml.

EXPERIMENTAL

Materials

I (UK-68,798) and the internal standard (I.S., UK-69,308) (Fig. 1) were prepared by Pfizer Central Research [1]. Other chemicals and reagents were of Analar or equivalent grade, except for *tert.*-butyl metyl ether (Fisons, HPLC grade).

Preparation of solutions

Stock solutions of I and I.S. were prepared in methanol at a concentration of 500 μg/ml and stored at 4°C. Stock solutions were stable for several months. These solutions were diluted prior to use with methanol to concentrations of 5 μg/ml. Phosphate buffer (0.1 *M*) was prepared by dissolving 14.2 g disodium hydrogen orthophosphate in 1 l of water and adjusting pH to 7.4 using phosphoric acid (85%, *i.e.* 8.5 *M*). Ammonium phosphate buffer (20 m*M*) for the HPLC mobile phase was prepared by dissolving 2.3 g ammonium dihydrogen orthophosphate in 1 l of water and adjusting the pH to 7.0 using ammonia solution (35%, *i.e.* 20 *M*). Phosphoric acid solution, 0.02 *M*, was prepared by dissolving 0.14 ml of phosphoric acid (85%) in 100 ml of water.

Extraction procedure

Urine (1.0 ml), after addition of 20 μl of I.S. solution, was mixed with 1.0 ml of phosphate buffer and extracted with 5.0 ml of *tert.*-butyl methyl ether by rotary mixing at 30 rpm for 10 min. After centrifugation at 1730 *g* for 5 min the organic layer was transferred to a second tube and extracted with 1.0 ml of phosphoric acid (0.02 *M*). Following centrifugation at 1730 *g* for 5 min the organic layer was discarded and the aqueous layer neutralised with 2.0 ml of phosphate buffer. This was re-extracted with a further 5.0 ml of *tert.*-butyl methyl ether. After a final centrifugation at 1730 *g* for 5 min the ether was transferred to a tapered tube and evaporated to dryness under a stream of nitrogen. The dry residue was resuspended in 100 μl of the HPLC mobile phase and briefly mixed and centrifuged prior to transfer to an HPLC autosampler vial for automated analysis. From each sample 80 μl were injected onto the HPLC analytical column.

High-performance liquid chromatography

Urine samples prepared according to the above procedures were analysed by an HPLC system employing a Spherisorb 5-μm silica column (25 cm × 4.6 mm I.D., Hichrom, Reading, UK). The samples were injected by an ISS-100 autosampler (Perkin Elmer, Beaconsfield, UK) and eluted with acetonitrile–ammonium phosphate buffer, pH 7.0 (35:65, v/v) at a flow-rate of 1.0 ml/min. The elution was carried out at ambient temperature and column effluent was quantified using a Shimadzu SPD-6A UV spectrometer (Dyson Instruments, Hetton, UK) with detection at 230 nm. The chromatograms were integrated using a MULTICHROM chromatography data management system (VG Laboratories Systems, Altrincham, UK).

Preparation of calibration curves

A calibration curve was constructed with six different urine standards covering a range of 2.5–50 ng/ml for each assay run. The peak-height ratio (drug/internal standard) was calculated for all samples. The calibration curve 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 line parameters and correcting for any dilution used. Samples in which the concentration exceeded 50 ng/ml were either diluted with control urine prior to analysis or analysed over a higher concentration range. A maximal twenty-fold calibration range was used in any single assay run.

RESULTS AND DISCUSSION

Selectivity

Typical chromatograms for control blank, spiked human urine and dosed human urine are shown in Fig. 2. The retention times of I and I.S. are typically 8.5 and 10 min, respectively, although column age caused some variation. The N-desmethyl metabolite of I, which has been identified in human urine at concentrations less than one tenth that of parent drug, has a retention time of approximately 7.5 min on this system. Other identified metabolites of I do not extract by this method. Thus under the extraction and chromatographic conditions described here, no endogenous components or metabolites of I present in human urine were shown to interfere with the assay. In approximately 2% of volunteers, a late-eluting component was observed which required a run time of 30 min to avoid interference with subsequent sample runs.

Recovery

The recoveries of I and I.S. from urine were assessed at each stage of the extraction procedure. The recoveries following the first extraction with *tert.*-butyl methyl ether were 60 and 40%, respectively for 50 ng of I and 100 ng of I.S. Back-extraction from *tert.*-butyl methyl ether into 100 μl of phosphoric acid

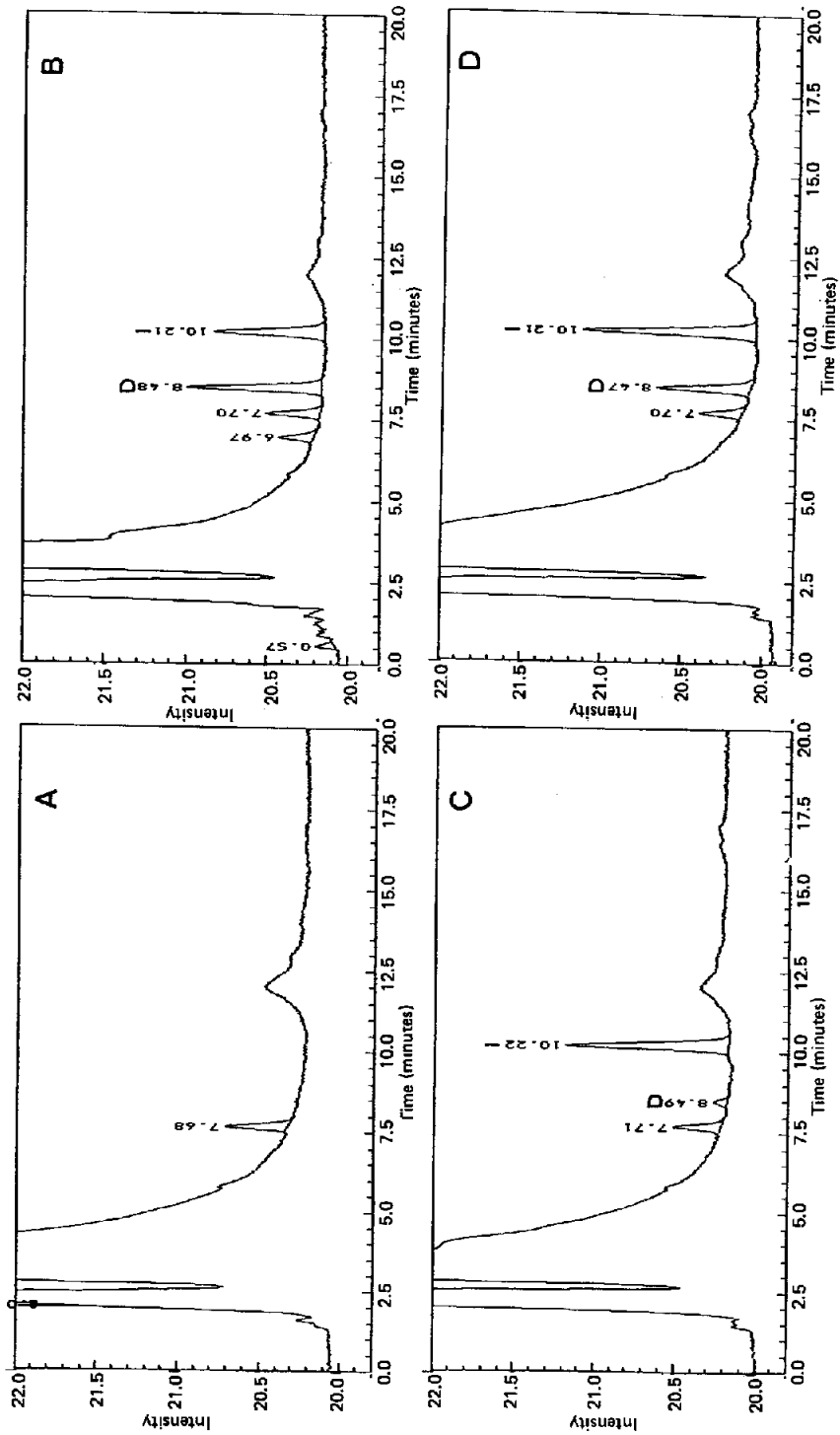

Fig. 2. Typical chromatograms showing (A) drug-free control urine extract, (B) control urine extract containing 50 ng/ml I and 100 ng internal standard, (C) control urine extract containing 2.5 ng/ml I and 100 ng internal standard and (D) urine extract containing 100 μl urine (220 ng/ml) from a human subject collected 0–24 h following a single oral dose of 0.5 mg I. Peaks: D = I (retention time 8.5 min); I = internal standard (retention time 10 min).

solution, 0.02 *M* (a lower volume was used to allow direct injection onto the analytical column), resulted in recoveries of 40 and 30%, respectively, from the original urine sample. The recoveries for the overall procedure including the second extraction into *tert.*-butyl methyl ether gave recoveries of 25 and 12%, respectively. Although it was possible to determine the recovery at each stage using the HPLC system described, the additional extraction stages were required to provide adequate clean-up in some urine samples and were therefore routinely employed for all analyses.

Despite the low recovery of analyte by the three-stage extraction procedure the method provides reproducible results and yields the best clean-up of samples. During method development, procedures giving higher recoveries gave lower precision and accuracy values due to interference in chromatography from endogenous materials. Recovery was not improved at higher pH, in keeping with the pK_a of I (7.0). Higher pH has also been shown to chemically reduce the potential N-oxide metabolite of I back to parent compound and was therefore avoided. Extraction of I is possible using solid-phase extraction but this was found to provide inadequate sample clean-up.

Precision and accuracy

Precision of the method was determined by analysing six identical samples of control urine containing I at concentrations of 5, 25 and 50 ng/ml (Table I). The coefficient of variation was less than 6%.

TABLE I

PRECISION OF DETERMINATION OF I IN HUMAN URINE

Added concentration (ng/ml)	Found concentration (mean ± S.D., $n = 6$) (ng/ml)	Coefficient of variation (%)
5.0	5.1 ± 0.17	3.4
25.0	26.4 ± 0.72	2.7
50.0	51.7 ± 3.02	5.8

The concentration of I in control urine samples to which drug had been added at concentrations unknown to the analyst were determined. For nine samples analysed the mean deviation ($n = 9$) was 9.5 ± 7.3%. The day-to-day performance of the assay was monitored by analysis of quality control samples at low, medium and high concentrations within the assay range. Data were deemed acceptable if the mean deviation of the concentrations determined in these samples was less than 10%.

Linearity and limit of determination

Calibrations obtained by plotting peak-height ratio (drug/internal standard) *versus* concentration were linear over the range 2.5–50 ng/ml. Linear calibration was also obtained over the range 50–1000 ng/ml. A maximal twenty-fold calibration range was used for any single sample batch to avoid the need for weighting of regression data. The lowest standard (2.5 ng/ml) was set as the limit of determination of the assay as this provided adequate sensitivity for that required in sample analysis.

The use of unbonded silica gel with reversed-phase eluents has previously been shown to be of value in the analysis of basic amines [4,5]. In the case of I it provides greatly improved peak shape, and hence a lower limit of determination, than that obtained using more standard reversed-phase HPLC methods.

Clinical sample analysis

This assay procedure has been used extensively for studies with I in human volunteers. The assay has proved robust and reliable during these analyses. Compound I shows significant renal clearance in healthy volunteers. A mean of approximately 60% of the drug is recovered unchanged in the urine, largely during the first 24 h, following single intravenous (0.5–10 μg/kg) and oral (1–12.5 μg/kg) doses. The similar renal excretion observed after oral and intravenous administration indicates the complete oral bioavailability of I.

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REFERENCES

1 P. E. Cross, J. E. Arrowsmith, G. N. Thomas, M. Gwilt, R. A. Burges and A. J. Higgins, *J. Med. Chem.*, 33 (1990) 1151.
2 M. Gwilt, H. W. Dalrymple, R. A. Burges, K. J. Blackburn, J. E. Arrowsmith, P. E. Cross and A. J. Higgins, *J. Mol. Cell Cardiol.*, 21 (Suppl. II) (1989) S11 Abstract.
3 D. K. Walker, G. W. Aherne, J. E. Arrowsmith, P. E. Cross, B. Kaye, D. A. Smith, D. A. Stopher and W. Wild, *J. Pharm. Biomed. Anal.*, 9 (1991) 141.
4 B. A. Bidlingmeyer, J. K. Del Rios and J. Korpi, *Anal. Chem.*, 54 (1982) 442.
5 R. J.-Y. Shi, L. Z. Benet and E. T. Lin, *J. Chromatogr.*, 377 (1986) 399.