Journal of Chromatography, 615 (1993) 117–126
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6780

Method for the analysis of S9788, a drug to reverse resistance to anticancer agents, in animal plasma and human plasma and serum by high-performance liquid chromatography with ultraviolet detection

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(First received November 3rd, 1992; revised manuscript received February 9th, 1993)

ABSTRACT

An extraction method has been developed using benzene sulphonyl cation-exchange sample preparation cartridges and reversed-phase high-performance liquid chromatography with ultraviolet detection for the measurement of S9788, a drug to reverse resistance to anticancer agents, in plasma and serum. This includes a toxicokinetic assay which has a mean precision and accuracy of 11.7% and 7.9%, respectively, over the range 10–1000 ng ml^{-1} and a quantification limit of 10 ng ml^{-1} and a more sensitive pharmacokinetic procedure with a mean precision and accuracy of 5.0% and 7.9%, respectively, over the range 1–500 ng ml^{-1} and a quantification limit of 1 ng ml^{-1}. The specificity of the procedure has been demonstrated by mass and ultraviolet spectrometry, and linearity, precision, accuracy, recovery and sensitivity have been established. The assays have been successfully applied to toxicokinetic and pharmacokinetic studies.

INTRODUCTION

S9788, I, 6-[4-[2,2-di-(4-fluorophenyl)-ethylamino]-1-piperidinyl]-N,N′-di-2-propenyl-1,3,5-triazine-2,4-diamine, is a novel compound under development by Servier Labs. as a potential adjunct therapy in cases of multiple drug resistance (MDR) in cancer chemotherapy. MDR is observed with many chemotherapeutic agents including the anthracyclines, actinomycin D, vinca alkaloids and the epipodophyllotoxins [1], and appears to be associated with over expression of a membrane P-glycoprotein. This glycoprotein is usually referred to as P-gp or P170 and is believed to function as an ATP-dependent efflux pump for cytotoxic agents. The increased activity of this protein is thought to lead to a decrease in the intracellular concentration of the cytotoxic agent and, hence, decreased efficacy [2–4]. There are many classes of compounds which are thought to inhibit P-gp and/or to reverse MDR both *in vitro* and *in vivo*, but these generally have been shown to be toxic at the effective concentration for MDR reversal *in vivo* [5]. Compound I does not belong, structurally or functionally, to any of the other classes of compounds known to reverse MDR. The compound, unlike the other agents, has no other known pharmacological activity

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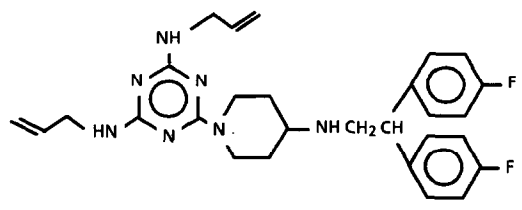
0378-4347/93/$06.00

and has shown no toxicity in rat and dog at doses which are known to be active non-cytotoxically *in vitro*.

In order to have a better understanding of the nature of the compound's activity, assay methods are required for toxicokinetic, pharmacological and pharmacokinetic studies. The objective of the analytical method development was to produce a method for the measurement of I in plasma and serum of sufficient simplicity to enable automation of the method for use in toxicokinetic studies, but also of sufficient sensitivity for use in pharmacokinetic analysis. The analytical method described herein is based on a solid-phase sample preparation procedure, comprising cation exchange as the primary attachment and dispersion forces as the secondary, to provide a selectively enriched sample with a minimum of endogenous interferences. Chromatographic separation is by reversed-phase HPLC with suppressed cationic interaction.



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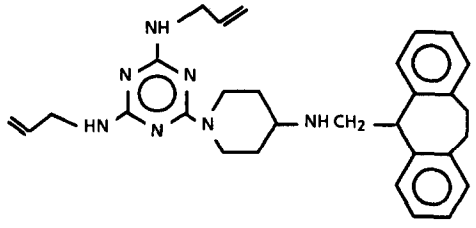

Internal Standard

Fig. 1. Structures of I and internal standard (S15061).

EXPERIMENTAL

Materials

Compound I and the internal standard (Fig. 1) were obtained from Technologie Servier (Orléans, France). Potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, orthophosphoric acid and ammonia (0.88 SG) were of Aristar grade and obtained from BDH (Poole, UK). Methanol, acetonitrile and triethylamine were of SpS quality and obtained from Romil (Loughborough, UK). Deionised water was prepared on site and further purified using an Elga UHQ system (Elga, High Wycombe, UK). Bond Elut solid-phase cartridges were the benzene sulphonyl functionality (SCX) and were purchased from Varian International (Harbor City, CA, USA). Plasma or serum was prepared from freshly heparinised or coagulated blood, as appropriate, from healthy volunteers. Control animal plasma (mouse, rat, dog and monkey) was obtained from the Toxicology Unit, Biologie Servier (Gidy, France). Reference patient plasma was obtained from a cancer unit in Paris, France.

Preparation of standards

A stock solution was prepared in which I was dissolved in methanol and diluted to a final concentration of 2.5 mg ml^{-1}. Secondary solutions were prepared by diluting aliquots of this solution to give standards in methanol ranging from 1 to 300 $\mu g\ ml^{-1}$. Aliquots (100 μl) of these secondary solutions were added to control plasma or serum to give final concentrations of I of 0.5–1000 ng ml^{-1}. These standards were stored, together with control matrix, at −20°C until use. Calibration, validation and quality control standards were independently prepared from separate stock solutions.

Chromatographic system

Analysis was performed on a Hewlett Packard HP1050 liquid chromatograph, consisting of a pump, autosampler, column oven and UV detector (Bracknell, UK). Separations were carried out under isocratic conditions on a Hypersil ODS (3 μm) column (15 cm × 0.46 cm I.D.) purchased from Shandon Scientific (Runcom, UK). The

mobile phase was comprised of 25 m*M* KH_2PO_4 (349.5 ml), adjusted to pH 4.0 with orthophosphoric acid, triethylamine (0.5 ml) and acetonitrile (650 ml). Optimal separation was achieved at 0.75 ml min^{-1} at an operating temperature of 40°C. Detection was by UV absorbance at 219 nm for the toxicokinetic method and at 230 nm for the more sensitive pharmacokinetic method.

Extraction of I from plasma or serum

The samples were prepared for analysis by solid-phase extraction. The frozen matrix was thawed at ambient temperature and centrifuged to remove fibrin, formed as a result of freezing and thawing.

Method for pharmacokinetic analysis

To a 1-ml plasma or serum sample was added an aliquot (50 μl) of the internal standard in methanol (600 ng ml^{-1}) and 500 μl of 25 m*M* KH_2PO_4 (pH 3.0). The Bond Elut (SCX) cartridge (50 mg) was solvated with methanol (1 ml) and conditioned with 25 m*M* KH_2PO_4 buffer (pH 3.0, 1 ml) prior to loading the plasma–buffer mixture. After loading, the cartridge was washed sequentially with a mixture of 50 m*M* KH_2PO_4 (pH 4.0) and acetonitrile (70:30, v/v, 0.5 ml), water (0.5 ml) and methanol (0.5 ml). Elution of I and internal standard was in a 2% (v/v) ammonia in methanol solution (0.5 ml). The extracts were evaporated to dryness under vacuum in a centrifugal evaporator (Life Science Industries, Dunstable, UK), and were reconstituted in a mixture of mobile phase and water (50:50, 150 μl), prior to injecting an aliquot (25 μl) onto the HPLC system.

Method for toxicokinetic analysis

To each 1-ml sample was added an aliquot (50 μl) of internal standard in methanol (2 μg ml^{-1}) and 25 m*M* KH_2PO_4 (pH 3.0, 0.5 ml). The Bond Elut (SCX) cartridges (100 mg) were solvated with methanol (1 ml) and conditioned with the 25 m*M* KH_2PO_4 buffer (pH 3.0, 1 ml). The sample–buffer mixture was loaded slowly onto the sorbent over a 2-min period. The cartridges were washed sequentially with a mixture of 50 m*M* KH_2PO_4 (pH 4.0) and acetonitrile (70:30, v/v, 1 ml), methanol (1 ml), water (1 ml), and a mixture of 50 m*M* K_2HPO_4, pH 7.0 and acetonitrile (35:65, v/v, 0.4 ml). Elution of I and internal standard was achieved by the addition of a further aliquot (0.5 ml) of the final wash mixture, a 20-μl portion of which was injected directly onto the HPLC system.

Validation of the methods

Intra-assay variability was assessed from ten plasma or serum validation replicates, once matrix compatibility had been demonstrated, of standards at nominal concentrations ranging from 0.5 to 500 ng ml^{-1} for the pharmacokinetic method and from 3 to 1000 ng ml^{-1} for the toxicokinetic assay. The concentration of I was calculated by reference of the peak-height ratios (I/internal standard) against an independently prepared set of calibration standards which were co-analysed in duplicate with each analytical run.

Regression analysis was used to assess the closeness of the fit of the observed data to the actual concentrations by regressing the measured concentrations of the intra-assay results against the actual concentrations. Using the data from this validation procedure the limit of quantification of the method was established as the lowest concentration which had a signal-to-noise ratio of at least 3:1, and had precision and accuracy values less than 20%.

Determination of specificity

The specificity of the analytical procedure was demonstrated by MS of fractions of the HPLC eluate. Human plasma containing I at *ca.* 25 μg ml^{-1} and control plasma were extracted as previously described, and an aliquot of each was injected onto the HPLC system. A timed fraction, corresponding to the I peak as determined by injection of a chemical standard, was then collected. The fractions were evaporated to dryness and reconstituted in a minimal volume of acetonitrile for MS analysis using a Nermag R10-10C quadrupole mass spectrometer controlled by a Sidar Series 2026 data system (Delsi Nermag UK, JS Agency, Altrincham, UK).

A 2-μl aliquot of the reconstituted sample was analysed using the direct insertion probe in the desorption chemical ionisation (DCI) mode. Ammonia reagent gas was used, with an indicated source pressure of 13.3 Pa and a temperature of 115°C. The DCI probe filament current ranged from 50 to 500 mA and was increased at a rate of 9 mA s^{-1}. Spectra were recorded over a mass range of 60–850 a.m.u. at 90 eV. The specificity was also determined by the use of a diode-array detector (Shimadzu SPD M6A, Dyson Instruments, Houghton-le-Spring, UK).

Recovery

The recovery of the extraction procedure was assessed using ^{14}C-radiolabelled I. A mixture of labelled and unlabelled I was added to human plasma to give three concentration levels: 20, 1000 and 20 000 ng ml^{-1}. Each of these samples was extracted using the method employed for toxicokinetic studies and an aliquot (100 μl) of every step was analysed by scintillation counting on a 2200CA counter (Canberra Packard, Pangbourne, UK).

Toxicokinetic studies

Male and female Wistar rats (two males and two females per dosing group) were given a single intravenous dose of I at either 4, 8 or 12 mg kg^{-1}. Blood (1.5 ml) was sampled at each time point from each animal over a 24-h period. Heparinised plasma was prepared by centrifugation and stored at −20°C until analysis.

Clinical studies

The pharmacokinetics of I were investigated during administration to cancer subjects (n = 3 per dose group), as part of a dose tolerance study, at single intravenous infusion doses ranging from 8 to 56 mg m^{-2} over a 30-min period. Blood (5 ml) was taken at predetermined times up to 48 h, and heparinised plasma was prepared by centrifugation and stored at −20°C until analysis.

RESULTS

Under the conditions described for the analysis of I in plasma or serum, the compound was well separated from the internal standard and from endogenous biological components (Fig. 2).

Over the ranges described for each assay, linearity was demonstrated with mean correlation coefficients >0.999 for the toxicokinetic procedure (n = 10, y = 0.024x + 0.013) and >0.9999 for the pharmacokinetic method (n = 11, y = 0.041x + 0.015).

Intra-assay precision and accuracy were determined from ten replicates at each concentration and for each assay procedure. Inter-day variability was assessed for a series of analytical runs, at three different concentrations, performed over

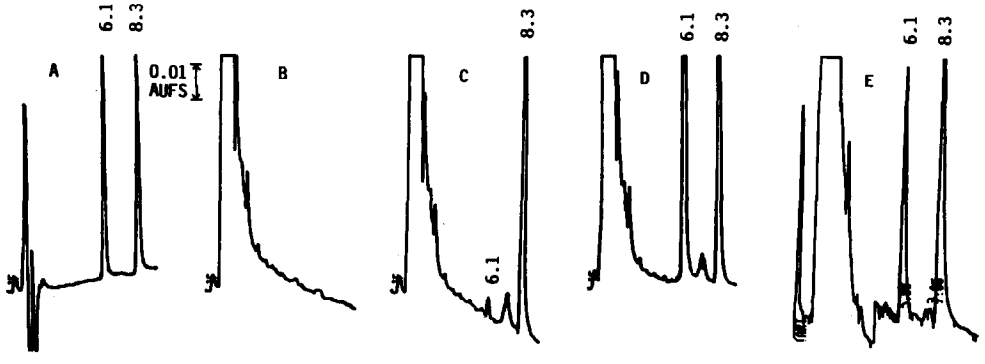

Fig. 2. Representative chromatograms. (A) Standards of I and internal standard at 6.1 and 8.3 min, respectively. Injection volume 25 μl. (B) Blank plasma extract indicating the absence of plasma interferences. (C) Plasma extract with I at 1 ng ml^{-1} and internal standard at 25 ng ml^{-1}. (D) Plasma extract with I at 100 ng ml^{1} and internal standard at 25 ng ml^{-1}. (E) 12-h Plasma extract of patient receiving I at 40 mg m^{-2}.

TABLE I

SUMMARY OF INTRA-ASSAY VALIDATION OF I IN HUMAN SERUM OVER THE RANGE OF 10–1000 ng ml^{-1}: TOXICOKINETIC METHOD

Theoretical concentration (ng ml^{-1})	n	Concentration found (mean ± S.D.) (ng ml^{-1})	C.V. (%)	Accuracy (%)
10.0	10	11.8 ± 0.8	7.1	+18
30.0	10	30.0 ± 3.6	12.1	−0.1
100	10	105 ± 10.9	10.5	+4.7
300	10	265 ± 45.4	17.1	−12
1000	10	952 ± 113	11.9	−4.7

several weeks. The results are summarised in Tables I–IV.

Based on the criteria of the lowest concentration which has a baseline signal-to-noise ratio greater than 3:1 and an accuracy and precision of measurement better than 20%, the limit of quantitation for the toxicokinetic assay was 10 ng ml^{-1} (C.V. = 7.1%, accuracy = +18%). The limit of quantitation for the pharmacokinetic assay was set at 1 ng ml^{-1} (C.V. = 13.6%, accuracy = +1.0%).

Recovery was assessed by the use of ^{14}C-labelled I spiked into plasma over a large concentration range (20–20 000 ng ml^{-1}) and using the toxicokinetic assay. Between 73 and 79% of I was recovered in the final eluent (mean 76.4%). Recovery was not concentration-dependent, indicating that the catridge was not saturated at the highest concentration. Less than 2% was lost in the wash phases, with the majority of the lost radioactivity (15–17%) present in the unretained plasma fraction and residual plasma from the container. The unretained fraction may be due to plasma protein binding.

TABLE II

SUMMARY OF INTRA-ASSAY VALIDATION OF I IN HUMAN PLASMA OVER THE RANGE OF 1.0–500 ng ml^{-1}: PHARMACOKINETIC METHOD

Theoretical concentration (ng ml^{-1})	n	Concentration found (mean ± S.D.) (ng ml^{-1})	C.V. (%)	Accuracy (%)
1.0	10	1.0 ± 0.1	13.6	+1.0
2.0	9	1.9 ± 0.1	5.2	−3.3
3.0	10	3.1 ± 0.4	13.6	−2.3
5.0	9	4.9 ± 0.1	2.7	−1.3
10	10	9.8 ± 0.4	3.8	−2.5
30	10	29.2 ± 0.5	1.7	−2.5
100	10	97.7 ± 1.1	1.1	−2.3
300	10	294 ± 5.5	1.9	−1.9
500	10	493 ± 6.1	1.2	−1.3

TABLE III

SUMMARY OF INTER-ASSAY VALIDATION OF I IN HUMAN SERUM: TOXICOKINETIC METHOD

Theoretical concentration (ng ml^{-1})	n	Concentration found (mean ± S.D.) (ng ml^{-1})	C.V. (%)	Accuracy (%)
30.0	10	31.5 ± 5.0	16.0	+5.0
100	10	98.7 ± 12.0	12.1	−1.3
1000	10	1018 ± 141	13.8	+1.8

Specificity

Reconstructed ion chromatographic (RIC) traces for the protonated molecular ion (m/z 506) from the HPLC fractions of spiked and control plasma were compared to the chemical standard (MW = 505). There was a prominent peak in the m/z 506 RIC trace for the fractions taken from the spiked plasma and the chemical standard. When spectra were taken for the peak in the m/z 506 RIC trace for the plasma fraction, a protonated molecular ion ([M+H] = m/z 506) was observed in the spectrum. Also present were a

TABLE IV

SUMMARY OF INTER-ASSAY VALIDATION OF I IN SERUM: PHARMACOKINETIC METHOD

Theoretical concentration (ng ml^{-1})	n	Concentration found (mean ± S.D.) (ng ml^{-1})	C.V. (%)	Accuracy (%)
3.0	7	2.7 ± 0.2	8.1	−8.5
30.0	7	29.3 ± 1.1	3.7	−2.3
500	7	492 ± 19.4	4.0	−1.6

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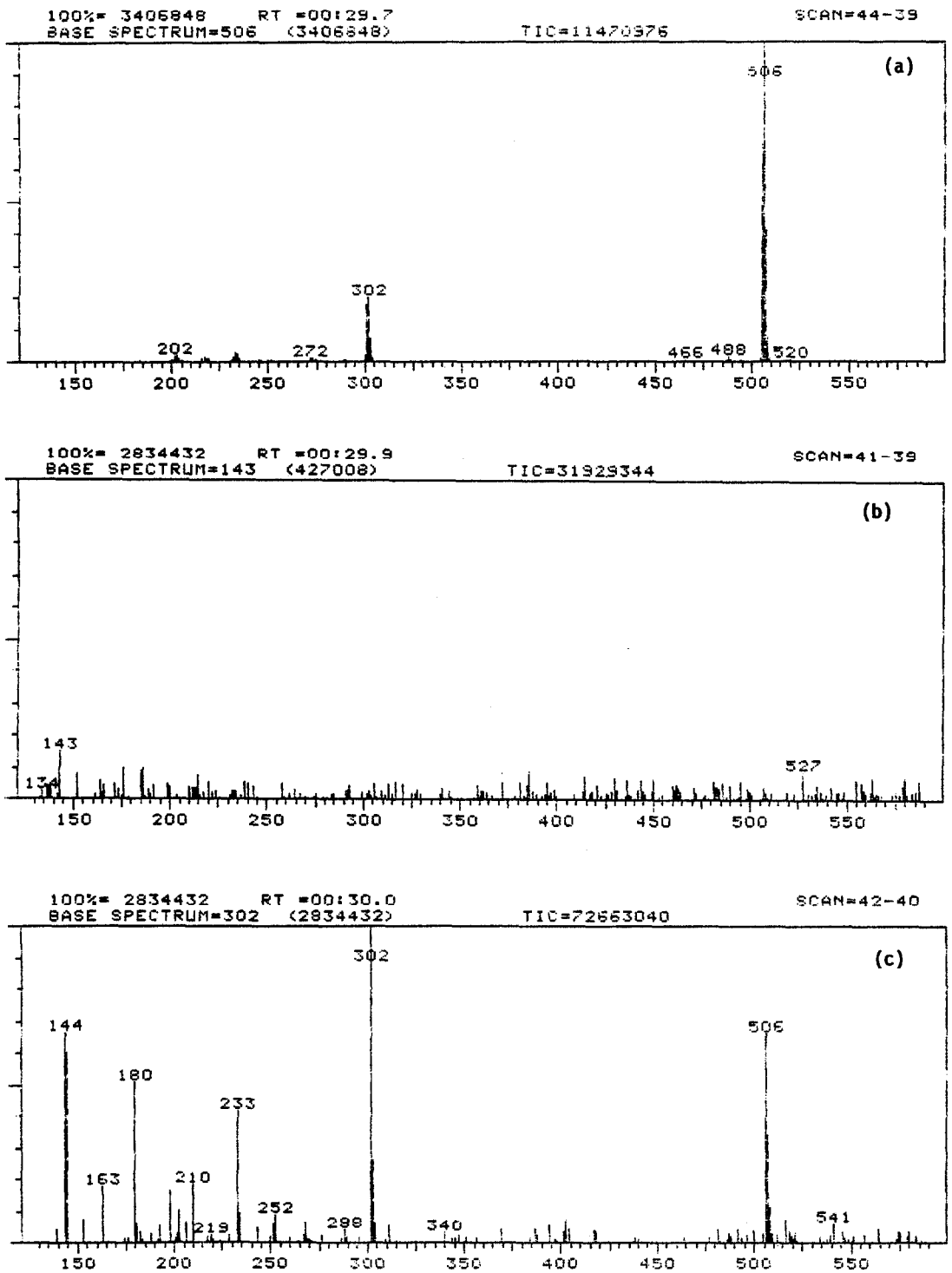

Fig. 3. Mass spectra of I. (a) Standard solution. (b) Control plasma. (c) Plasma extract containing I.

small m/z 203 fragment ion due to the bisfluorobenzhydryl moiety and a doubly protonated molecular ion of m/z 507 which has occasionally been observed with samples isolated from a biological matrix. The m/z 506 RIC trace obtained from the control plasma sample was of a reduced intensity and there was no significant peak observed (Fig. 3).

The spectral analysis obtained from the diode array showed identical UV absorbance spectra for the chemical standard and for the isoretentive peak observed in the spiked plasma sample.

The positive results obtained from both these specificity checks confirm the identity of the measured peak in this assay as I.

Matrix compatibility

Mouse, rat, dog and monkey plasma was applied to the toxicokinetic assay. The method was found to be suitable in terms of accuracy, precision and sensitivity when compared to human plasma. There were, however, differences in the slopes of the regression line obtained for each matrix, suggesting some disparity in relative recoveries of either I or internal standard between the various species. Whilst there were no differences between human heparinised plasma and serum, there was a marked difference between the slopes observed for human heparinised plasma and citrated plasma. This may perhaps be a result of the difference in the matrix pH and buffering capacity due to the two anticoagulants, such that the strength of attachment of I is reduced in the citrated medium.

Citrate and benzene sulphonate have similar strong cationic selectivity, and it may be that I first forms an ion pair with the citrate ions thus reducing the charge on the protonated sites available for interaction with the sulphonate bonded phase. This is evidenced by the fact that 27% of the compound is eluted in the buffer–acetonitrile wash from citrated material, but $<1\%$ is lost from the heparinised plasma.

Toxicological and clinical applications

When rats were given I as an intravenous dose and plasma samples analysed, the plasma profile up to 24 h (Fig. 4) showed a direct relationship between the area under the plasma concentration *versus* time curve to 24 h (AUC_{0-24}) and the dose of I administered. Similarly, when patients received I as a 30-min infusion at doses up to 56 mg m^{-2}, plasma concentrations of I were measurable for at least up to 24 h and the AUC_{0-24} increased with increasing doses (Fig. 5). The AUC_{0-24} in these patients, however, did not in-

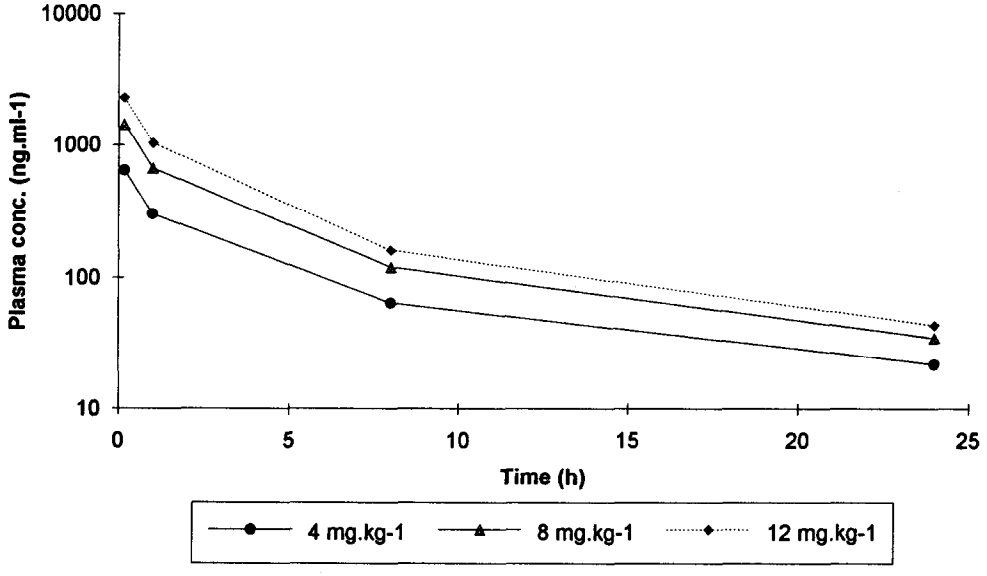

Fig. 4. Mean plasma levels of I in rats (n = 4 per dose group) following a single intravenous administration of the compound.

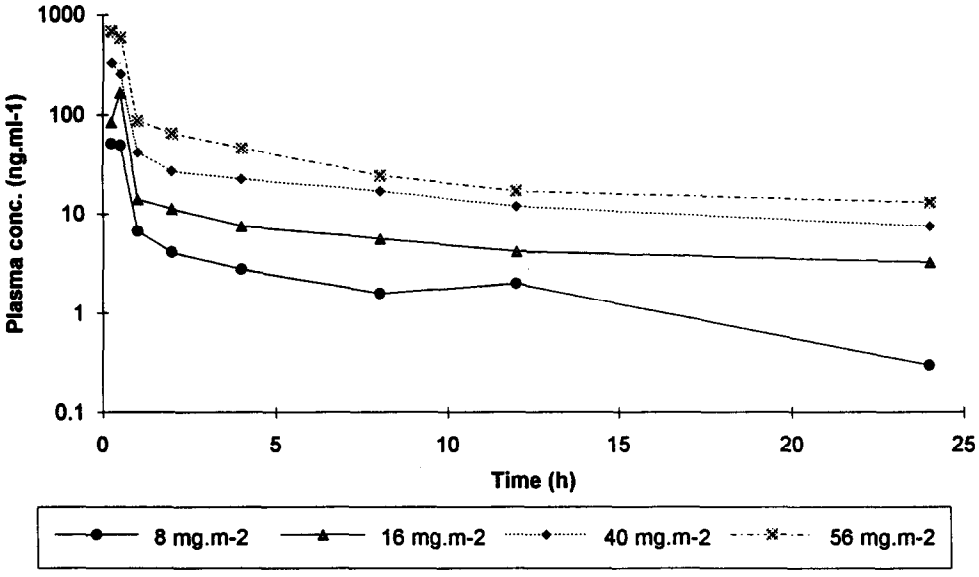

Fig. 5. Mean plasma levels of I in cancer subjects (n = 3 per dose group) following single intravenous infusions of I over 30 min.

crease in direct proportion to dose when compared to the rats (Table V).

DISCUSSION

Both I and the internal standard were well resolved from each other and from endogenous material within a 10-min run time illustrating the selectivity of the extraction and separation processes. However, a chromatographic peak was seen, of variable height, eluting between the analyte and internal standard. This was eventually traced to the HPLC borosilicate colourless glass vials (Chromacol, Welwyn, Garden City, UK). When the vials were washed with 2% (v/v) ammonia in methanol, the peak was reduced to insignificant levels.

Another potential source of interference was observed with the volumetric glassware in which methanolic solutions of I were prepared prior to spiking into plasma for the preparation of standards. It was found that there was a persistence of I adsorbed onto the surface of the glass, possibly by silanol interaction as was thought for the interference on the elution vials, even after normal laboratory washing procedures. This problem was also resolved by washing all laboratory glassware that was used for this assay in 2% (v/v) ammonia in methanol.

The fact that I is capable of such interactions via its secondary and tertiary amino groups as implied above is evidenced by the selected extraction method. Whilst the benzene sulphonyl group on the Bond Elut cartridge is capable of hydrophobic interaction through dispersion forces between the benzene ring on the support and the two fluorinated phenols on I, the main retention

TABLE V

SUMMARY OF THE MEAN AUC_{0-24} OF I IN HUMAN AND RAT FOLLOWING INTRAVENOUS ADMINISTRATION OF THE COMPOUND

Dose[a]	AUC_{0-24} (ng ml^{-1} h)	
	Human	Rat
4	–	2380 ± 264
8	60.2 ± 20.6	4991 ± 247
12	–	7426 ± 358
16	193 ± 70.5	–
40	576 ± 184.1	–
56	954 ± 9.9	–

[a] Human dose in mg m^{-2}. Rat dose in mg kg^{-1}.

mechanism is via the ionic bonds formed between the negative sulphonyl groups on the sorbent and the protonated nitrogens of I. These interactions are sufficient to retain I (pK_{a1} = 5.0; pK_{a2} = 6.8–8.0) and the internal standard under weakly acidic conditions (pH 4.0) in the presence of 30% (v/v) acetonitrile. Elution is achieved by the deprotonation of the analytes and the disruption of the dispersion forces under the basic environment induced by the methanol–ammonia mixture. The major ionic attachment is probably the moiety with the pK_a of 5.0, since elution is achieved in the toxicokinetic method at pH 7.0 in the presence of 65% acetonitrile, at which pH the moiety with the higher pK_a would still be mostly protonated. Such an approach provides a thorough clean-up of the plasma/serum samples and is particularly suited to analysis by reversed-phase HPLC since, in the case of the pharmacokinetic method, the eluate is easily evaporated.

Initial chromatograms indicated strong secondary interactions between I and free silanols on the silica support of the Hypersil column, evidenced by broad tailing of I and internal standard peaks, whereas the endogenous peaks exhibited reasonable symmetry. This phenomenon may have been overcome by the suppression of I ionisation by raising the pH of the mobile phase to 2 units above the pK_a of the analytes. This difficulty was overcome instead by the incorporation of the tertiary amine modifier, triethylamine, at 0.05% (v/v) into the mobile phase to saturate all available surface silanols. Therefore, retention is governed by the reversed-phase mechanism alone, resulting in a system efficiency of approximately 13 500 plates.

Assay specificity of I has been confirmed using MS analysis. It is interesting to note the 506/302 ratio differences of the spectra obtained for the chemical standard and the processed sample extract.

The samples were analysed using chemical ionisation (CI) MS which is a "softer" ionisation procedure than that obtained with electron impact (EI). The CI analysis results in a prominent protonated molecular ion for spectra A and C (the m/z 506 ion) with the cleavage/fragment ion (the m/z 302 ion) dependent on the CI ion source reaction which can be influenced by the presence of biological matrix ions in the sample extract (spectrum C). In general samples analysed by EI tend to give more reproducible ion intensities for fragment ions but can also result in weak or total absence of the molecular ion, thus not yielding molecular mass information for the compound being studied.

There is considerable overlap between the two assay ranges and it would seem more appropriate to combine both methods into a single assay for I. The toxicokinetic method was designed with a view to automation and it was therefore necessary for the elution matrix to be compatible with the mobile phase. A 20-μl injection of this matrix was found to be the optimal volume for peak symmetry and resolution from interferences. Based on knowledge of similar compounds, the predicted half life of I would require greater sensitivity and, therefore, the pharmacokinetic method was designed with high sensitivity as the main objective. In order to achieve this, evaporation of the eluting solvent and reconstitution in a hypoeluotropic medium with on-column band focusing was appropriate.

Both methods have been applied successfully to human and animal studies. For both assay procedures where plasma concentrations of I were beyond the calibration range of the assay, it was necessary to dilute an aliquot of sample in its corresponding control matrix in order to obtain a plasma concentration within the assay calibration range. It had been demonstrated that dilution of high-concentration samples with control matrix prior to extraction yielded similar results to the original values when back-calculated.

CONCLUSIONS

The use of a dual retention mechanism in the solid-phase extraction process coupled to the elimination of silanol interaction during chromatography has given rise to a highly sensitive, robust and selective analytical method for I which can be tailored for both toxicological and clinical applications.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. P. Bennett and Mr. M. Briggs for technical assistance and R. Brownsill for MS analysis.

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