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Determination of calcium acetylhomotaurinate in human plasma and urine by combined gas chromatography–negative-ion chemical ionization mass spectrometry

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ABSTRACT

A highly sensitive and specific assay has been developed for the determination of calcium acetylhomotaurinate and the internal standard (LM 3041) at the picomole level in human plasma and urine by gas chromatography–mass spectrometry with methane as the reagent gas. After a multiple-step extraction process, the cleaned-up organic extract was derivatized with pentafluorobenzoyl chloride at ambient temperature. Subsequently, chlorination followed by amidation of the sulphonic acid group led to the N-pentafluorobenzoyl di-*n*-butylamide derivatives. The mass spectrometer was set to monitor the abundant [M − HF] ions (*m/z* 424 and 438), which were generated in the ion source switched in the negative-ion chemical ionization mode. This assay required 1 ml of plasma or 50 μl of urine, and the detection limit was 1 ng/ml. The accuracy of the assay was tested day by day with quality control specimens spiked blind to the analyst. The mean difference between the theoretical and actual values was less than 8%.

INTRODUCTION

Calcium acetylhomotaurinate (Ca AOTA, I) is a synthetic compound derived from homotaurine, a reference γ-aminobutyric acid (GABA)-ergic agonist. Several studies have suggested that I reduces the voluntary ethanol intake in animals and humans [1,2], owing to a central stimulation of GABA-ergic receptors after crossing the blood–brain barrier [3]. To evaluate the behaviour of I in body fluids in therapeutic settings, a reliable and robust method is required.

Most of the amino acids in protein hydrolysates have been identified or measured in biological matrices by various techniques, such as colorimetric, fluorimetric and enzymic assays [4–6]. More recently, chromatographic procedures using an amino acid analyser or a high-performance liquid chromatographic (HPLC) system with UV or fluorescence detection have been widely used for the quantitative determination of amino acids as *o*-phthalaldehyde (OPA)-thiol or fluorenylmethoxycarbonyl (FMOC) derivatives.

An HPLC method with fluorimetric and electrochemical detection has been previously described for the measurement of I in the high nanogram to microgram range in dog plasma pretreated intravenously with a 100 mg/kg dose of I [7].

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Obviously this assay is not sensitive and specific enough to study the bioavailability and pharmacokinetics of new oral formulations in humans. Currently, high-resolution capillary gas chromatography–mass spectrometry (GC–MS) remains the method of choice for the quantitative determination of I in complex biological extracts down to 1 ng/ml.

We have developed a negative-ion chemical ionization (NICI) GC–MS assay with methane as the reagent gas to quantify with precision and accuracy the low levels of I following oral administration of the drug. This assay was performed with 1 ml of plasma or 50 μl of urine. The extracts were treated with pentafluorobenzoyl chloride (PFB-Cl) and di-*n*-butylamine (DBA). The derivatives gave rise to the intense and characteristic ions *m/z* 424 (I) and *m/z* 438 (internal standard), which were recorded by selected-ion monitoring. The feasibility of this new assay was demonstrated by preliminary results on the quantitative analysis of I in plasma of 24 healthy volunteers receiving an oral dose of 666 mg of I.

EXPERIMENTAL

Reagents

Calcium acetylhomotaurinate (I) and 4-acetylaminobutane sulphonic acid (LM 3041) as internal standard (I.S., II) were kindly supplied by Lipha (Lyon, France). All solvents were of analytical grade and were obtained from Merck (Darmstadt, F.R.G.). They were used without further purification. PFB-Cl, thionyl chloride and DBA were purchased from Aldrich (Strasbourg, France). Tetrabutylammonium hydrogensulphate ($TBA^+ H_2SO_4^-$) was obtained from Aldrich and was dissolved in 0.2 *M* sodium hydroxide at a concentration of 10%. Octadecyl (C_{18}) disposable extraction columns were supplied by J. T. Baker (Sochibo, France). All glassware was cleaned with an mechanical scaling brush, then left overnight in CrO_3–H_2SO_4 and finally rinsed with doubly distilled water. The PTFE caps of the tubes were also carefully cleaned to avoid any subsequent sample contamination.

Standard curves

Stock solution of I and II were prepared by dissolving each of the pure reference compounds in water to a primary concentration of 1 μg/ml. Secondary standard solutions obtained from appropriate dilutions in distilled water were carefully protected from light by aluminium foil and stored at 4°C until use.

Aliquots of 1 ml of drug-free human plasma were fortified with 50 μl of a 10 μg/ml I.S. solution in water, and various amounts of I ranging from 3.12 to 800 ng/ml. For urine samples (50 μl) the added amount of I.S. was the same, and the standard curve was elaborated in the same range as for plasma samples. The blank specimens were prepared in a similar way by spiking 1 ml of control plasma (or 50 μl of urine) only with the I.S. solution.

Extraction from plasma and urine samples

Plasma samples (1 ml), spiked as stated above with I.S., were placed in a small test-tube with 1 ml of acetonitrile. The mixture was carefully homogenized on a vortex mixer for 20 s, and the tubes were centrifuged at 1600 *g* for 15 min. The supernatant was transferred in a 10-ml screw-capped tube with 5 ml of methylene chloride. After agitation for 10 min with a reciprocating shaker, and centrifugation as described above, the aqueous layer was removed and subsequently hydrolysed with 0.2 ml of 10 *M* hydrochloric acid during 2 h at 100°C. Urine samples (50 μl) were directly hydrolysed without prior deproteinization. The hydrolysate was applied to a C_{18} disposable extraction column, previously conditioned with 1 ml of methanol followed by 1 ml of distilled water. The eluate was collected in a new 10-ml screw-capped tube and treated with 5 *M* sodium hydroxide to produce a strong basic solution.

Derivatization procedure

The derivatization procedure was adapted from the one previously described by Kataoka and co-workers [8,9] with minor modifications. The previous sample solution was supplemented with 20 μl of PFB-Cl and, after brief mixing at room temperature, the reaction mixture was washed twice with 3 ml of diethyl ether after adjustment to pH 3 with 2 *M* hydrochloric acid. After centrifugation, the organic layer was removed under vacuum using a Pasteur pipette. To the aqueous layer was then added 0.1 ml of 10% TBA^+ $H_2SO_4^-$ solution and 5 ml of methylene chloride. The tubes were shaken for 10 min at room temperature and then centrifuged at 1600 *g* for 10 min. The organic layer was transferred to another tube, and the solvent was evaporated under a gentle stream of nitrogen at 45°C. To the residue were added 50 μl of thionyl chloride, and the tubes were heated at 80°C for 30 min. To the reaction mixture was added 0.2 ml of 2 *M* DBA solution in acetonitrile.

The tubes were tightly capped and, after adjustment to acidic pH with 1 ml of 20% H_3PO_4 solution, the reaction mixture was extracted with 4 ml of pentane. The organic layer was transferred after centrifugation to another 10-ml screw-capped tube, and the solvent was evaporated under a stream of nitrogen. The residue was dissolved in 0.3 ml of ethyl acetate, and 1 μl of this solution was injected into the GC–MS system.

Precision and accuracy

To assess the precision and accuracy of the method, repeatability assays were carried out at four different levels (3.12, 25, 200 and 800 ng/ml). The spiked plasma samples were analysed the same day by the same analyst and, for each concentration level, coefficients of variation and mean percentages of error were calculated [10–12].

GC–MS analysis

The method was developed on a Hewlett-Packard 5985B gas chromatograph–mass spectrometer. GC was carried out on a fused-silica capillary column (25 m × 0.35 mm I.D., Chrompack), wall-coated with a OV 1701 liquid phase. The film thickness and inner side diameter of the column were 0.2 μm and 0.25 mm, respectively.

Samples were injected via a falling needle injector with helium used as the carrier gas (inlet pressure 0.7 bar). The injection port was maintained at 320°C. The oven temperature was raised from 240 to 310°C at 10°C/min, and the final temperature was held for 2 min.

One end of the capillary column was connected to the glass solid injector 1 mm from the bottom of the moving needle. The other end was directly introduced into the ion-source chamber of the mass spectrometer via a stainless-steel transfer line held at 310°C.

The HP 5985 GC–MS system was operated in the NICI mode with an electron energy of 100 eV, an emission current of 300 μA and an ion-source temperature of 150°C. Prior to the analysis, the instrument was tuned in the NICI mode using the fragments *m/z* 414, 595 and 633 from the perfluorotributylamine calibrant gas. The reagent gas methane was admitted via a gas-flow controller to an indicated ion-source pressure of 0.8 Torr.

The NICI mass spectra of N-PFB-DBA derivatives of I and II were recorded during a GC run by scanning repetitively the quadrupole mass filter every 0.9 s from *m/z* 100 to 500.

Quantitation of I was performed by focusing the instrument in the single-ion monitoring mode in order to measure the fragments *m/z* 424 and 317 (I) and *m/z* 438 and 311 (I.S.). The dwell time was equal to 50 ms for each mass range. The HP 5985B mass spectrometer was operated under the RTE 4/VM data operating system.

Quality controls

Throughout the study quality controls were realized blind to the analyst by spiking blank plasma samples with known amounts of I. These samples were assayed each day of the assay together with the "real life" biological samples collected during the pharmacokinetic study. After decoding the results, the absence of any systematic bias in the assay of I was controlled. For that reason, a regression analysis was carried out by plotting the found values *versus* the theoretical ones. The slope and intercept were than statistically compared to the theoretical values 1 and 0, respectively, using a Student's *t*-test.

Drug administration

Twenty-four young healthy volunteers (19–35 years of age) were examined and found to be in good health through medical history, physical examination and routine laboratory profiles. Each subject received a single oral dose of 666 mg of I

as tablets. Blood samples (5 ml) were collected in heparinized tubes at 0, 1, 2, 3, 4, 5, 6, 8, 12, 18, 24, 30, 36, 48, 60 and 72 h following the oral administration of the drug. Urine was collected as fractions corresponding to time intervals: 0–4, 4–12, 12–24, 24–36, 36–48, 48–72 and 72–96 h after the oral administration of two I tablets.

RESULTS AND DISCUSSION

Sample pretreatment and analysis

Compound I was prone to desacetylation during the sample pretreatment and the derivatization step. The complete hydrolysis of I to homotaurine prior to the extraction procedure overcame this non-controlled desacetylation process. After that, the N-PFB derivative was successfully made in order to obtain a reaction product with a high response under NICI conditions. On the other hand, the free sulphonic acid group prevented the above N-PFB derivative from organic solvent extraction. This problem was solved by ion-pair extraction with tetrabutylammonium hydrogensulphate.

Moreover, the extract must be carefully purified from endogenous substances that could interfere with the selected-ion monitoring determination of I. The multiple-step extraction process led to a "clean" residue devoid of most of the impurities. Owing to the use of a glass solid injector, and despite this rather long sample pretreatment, the laboratory throughput can be increased to 50 samples per day. Indeed, the moving needle injection technique, which is very protective for the capillary column, allowed an initial oven temperature much higher than that required for other sample inlets, such as splitless or on-column injectors.

In our analytical conditions, the retention times of I and II were 5.5 and 6.2 min, respectively. The two chromatographic peaks approximated a Gaussian shape curve, and each derivative was totally eluted from the column in less than 4.5 s. The peak width calculated at half height was *ca.* 1.2 s. A dwell time of 50 ms per mass led to a minimum of 30 measurements for each analyte.

Derivatization procedure

As stated by Kataoka and co-workers [8,9], the hydrolysis product of I totally reacted at the ambient temperature with PFB-Cl. Subsequently the chlorination of the N-PFB derivative described in Experimental was accomplished within 30 min at 80°C. The reaction of PFB homotaurine sulphonyl chloride with DBA proceeded rapidly to give the sulphonamide derivative without heating. The reaction product was extremely stable even over a long time. When the same plasma extract spiked with 25 ng/ml I was injected repeatedly during one month the coefficient of variation (C.V.) was 3.7% for 21 determinations.

The NICI mass spectra of N-PFB-DBA derivatives of I and II obtained with methane as the reagent gas exhibited characteristic ions in the high-mass region (Fig. 1). The loss of one 20 unit fragment, [M − HF], from the molecular ions gave

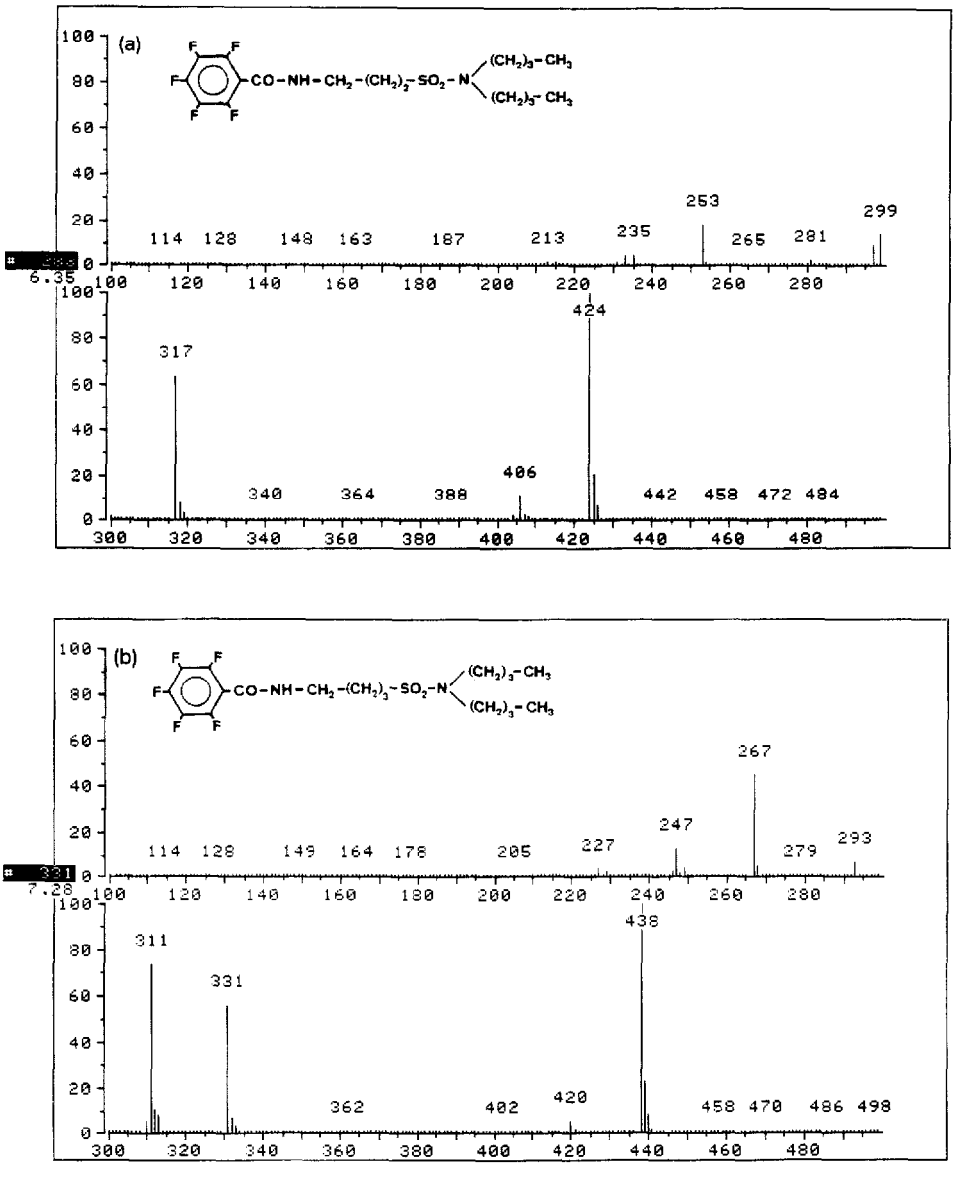

Fig. 1. Methane NICI mass spectra of the pentafluorobenzoyl di-*n*-butylamide derivatives of pure I (a) and LM 3041 (internal standard) (b).

rise to the base peaks observed at *m/z* 424 (I) and 438 (II). These two ions successively undergo elimination of the dibutylamine substituent and α cleavage of the sulphonamide function, leading to the formation of two groups of ions: *m/z* 317 and 253 for I and *m/z* 331 and 267 for II.

TABLE I

PRECISION AND ACCURACY OF THE ASSAY PROCEDURE

Theoretical concentration (ng/ml)	*n*	Observed concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Error (%)
800	10	798.43 ± 30.34	3.8	−0.2
200	10	204.64 ± 8.81	4.3	+2.3
25	10	25.91 ± 1.48	5.7	+3.6
3.12	10	3.115 ± 0.24	7.8	−0.3

Standard curves

The calibration graphs obtained each day of the assay by plotting the peak-area ratio *m/z* 424 and 438 *versus* the known plasma concentrations of I were straight lines ($r = 0.999976$) over the concentration range 3.12–800 ng/ml. The least-squares regression analysis passed near the origin with a mean intercept value close to zero ($n = 35$).

Precision, accuracy and limit of detection

The extraction yield was *ca.* 80%, and all sources of variability were considerably reduced by the use of a homologue as the I.S. The chemical behaviour of

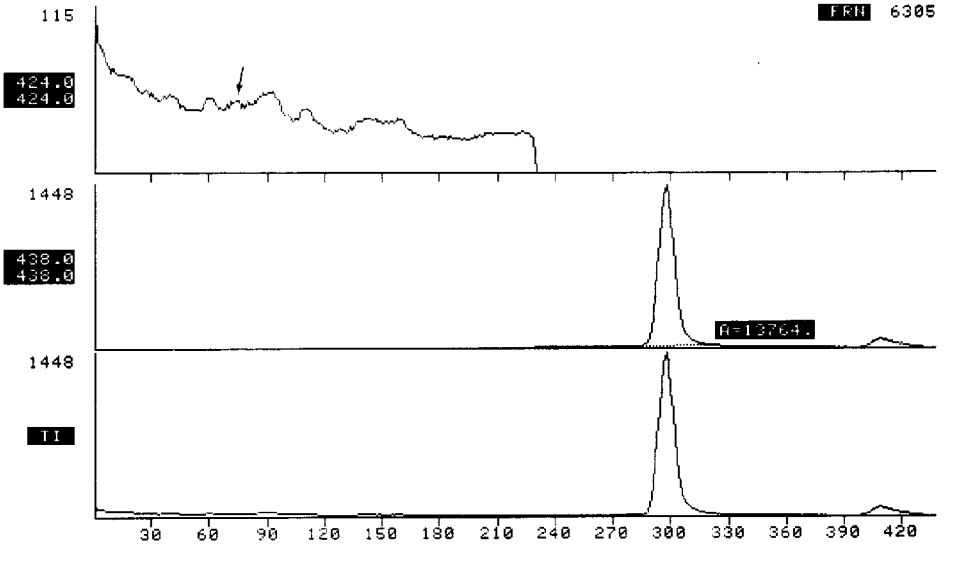

Fig. 2. Typical mass chromatogram obtained from a 1-ml blank plasma sample spiked with 500 ng/ml LM 3041.

this compound was quite close to that of I particularly during the deproteinization, hydrolysis and derivatization steps. The C.V. were less than 7.9%, and the mean percentage error ranged from −0.3 to +3.6% for plasma concentrations of I tested between 3.12 and 800 ng/ml (Table I).

As no signal was observed in the ten different blank plasma samples assayed in

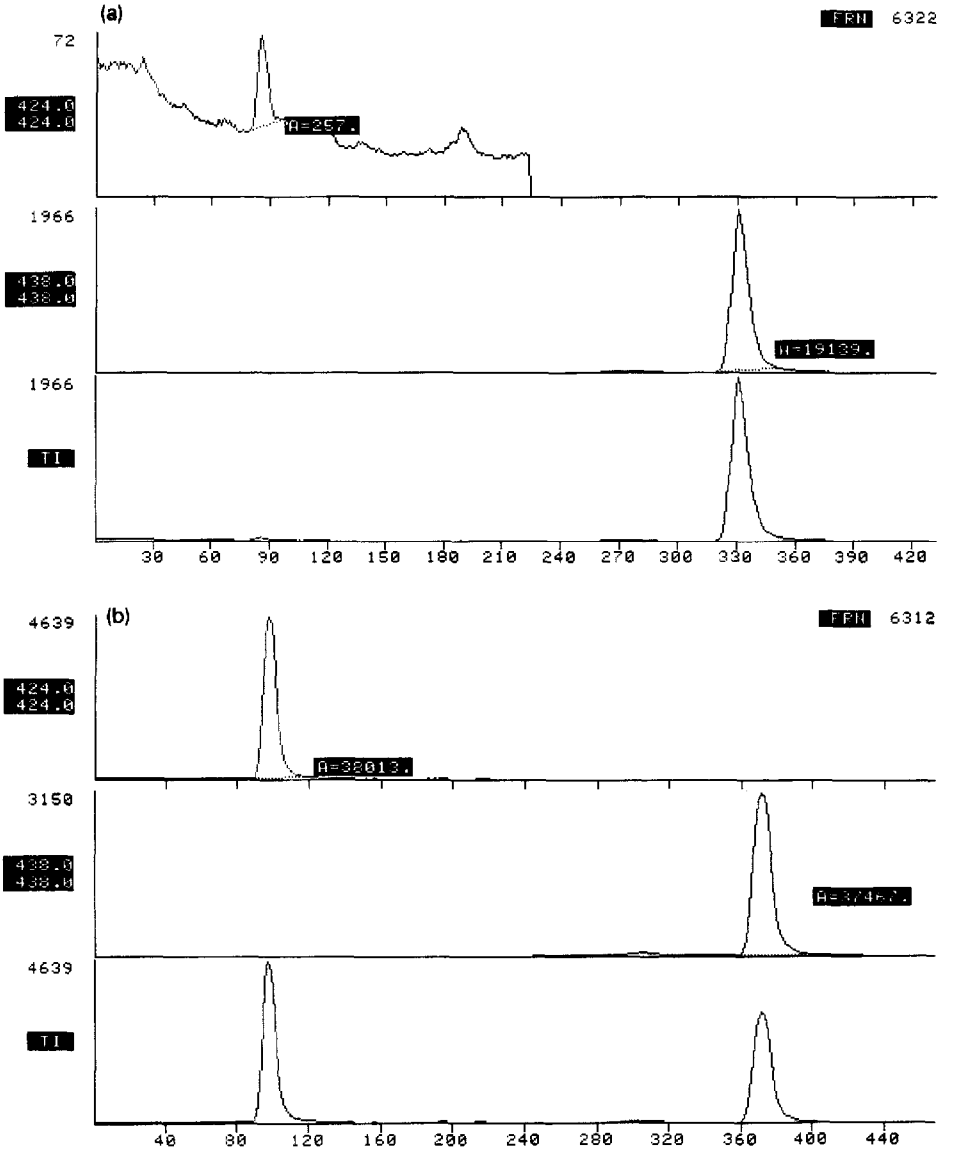

Fig. 3. Selected-ion monitoring traces obtained from a plasma sample fortified with (a) 3.12 ng/ml and (b) 200 ng/ml I and 500 ng/ml LM 3041.

the validation procedure, the limit of detection was tested at 3.12 ng/ml (ten determinations). The C.V. values (7.8%) and mean percentage error (−0.3%) clearly validated this concentration as the limit of quantification of the method.

A typical mass chromatogram obtained after the GC–MS analysis of a blank plasma sample is shown in Fig. 2, and that of control specimens spiked with 3.12 or 200 ng/ml are shown in Fig. 3. There was no interference from endogenous compounds in the single-ion monitoring trace of the fragment *m/z* 424. Thus quantitative measurements of the drug can easily be achieved for plasma concentrations of I of *ca.* 1 ng/ml. When a plasma extract corresponding to the limit of detection of the method was run, the signal measured at the retention time of I was equal to *ca.* 50 fmol injected into the GC–MS system.

Quality controls

Quality control analysis ($n = 105$) has shown that found concentrations correlate well with the theoretical ones ($r = 0.99677$) (Fig. 4). A Student's t-test was carried out in order to demonstrate that the slope (0.99674) and intercept (0.47758) values of the regression analysis were not significantly different from the theoretical values 1 and 0 at a probability level of 95% for $n-2$ degrees of freedom.

The t_{slope} value of − 0.4909 and the $t_{intercept}$ value of 0.2033 were lower than the limit (1.960) of the t_{table}. Consequently this assay has been applied to the

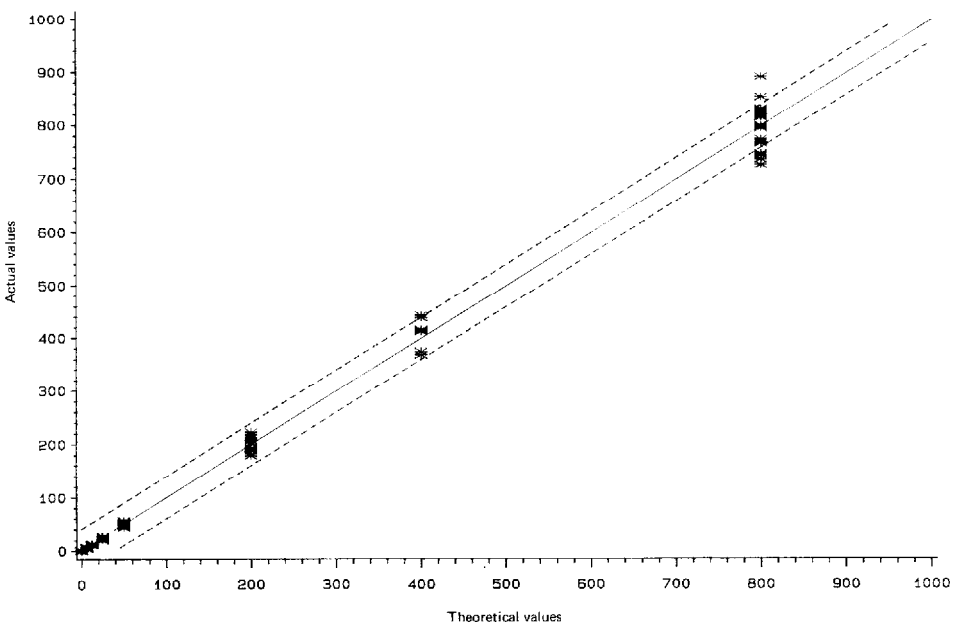

Fig. 4. Regression plot of the 105 quality control plasma samples analysed during the pharmacokinetic study.

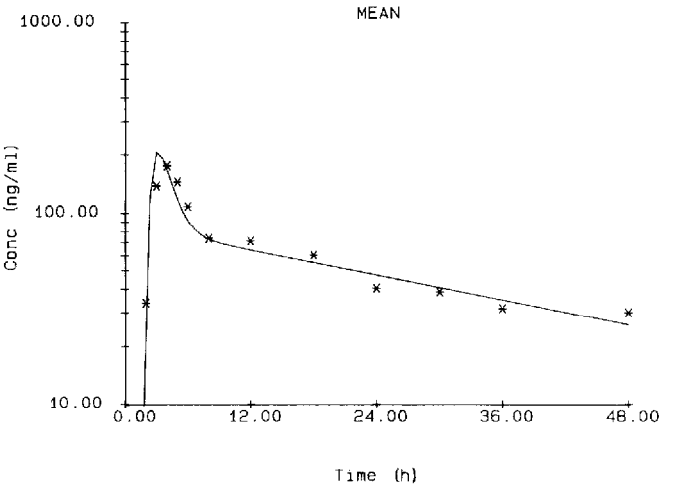

Fig. 5. Mean plasma concentrations of I *versus* time after a single 666-mg oral dose of I administered as two tablets to 24 healthy volunteers.

quantitative measurement of I without any constant or proportional systematic error. The mean difference between the theoretical and actual values was 8.0% for plasma concentrations ranging from 0 to 800 ng/ml.

Pharmacokinetic data

The applicability of this method was shown by the quantification of plasma concentrations of I in 24 healthy volunteers after oral administration of two tablets each containing 333 mg of I: a typical arithmetic plot of mean plasma concentrations ($n = 24$) *versus* time is presented in Fig. 5.

Pharmacokinetic analysis indicated that the mean C_{max} value was 180 ng/ml and that most of the drug was absorbed within 4 h. The rate constant (k_e) and the corresponding half-life ($t_{1/2}$) were calculated by linear least-squares fit of data points in the terminal slope (time *versus* log concentration). The mean $t_{1/2}$ value was 27.6 h. The total area under the I plasma concentration *versus* time curve ($AUC_{0-\infty} = 3700$ ng/ml/h) was calculated by the linear trapezoidal rule.

CONCLUSION

The GC–MS assay described in this paper afforded a sensitive and specific technique for the measurement of I at the picomole level in plasma and urine. The N-PFB-DBA derivative of I gave an intense signal and when the fragment m/z 424 was monitored under NICI conditions the detection limit of this assay was 1 ng/ml. The C.V. values and the mean percentages of error calculated during the different repeatability assays have demonstrated that, even around this limit of detection, the precision and accuracy of the method were suitable for routine

analysis of I. This was confirmed by the results obtained after the GC–MS measurement of 105 quality control specimens analysed blind to the analyst.

This technique has been successfully applied to a large batch of samples (more than 2500) over a long period of time. This assay was routinely carried out for pharmacokinetic and bioavailability studies following oral or intravenous administration of I.

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