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Quantitative measurement of a new synthetic hetrazepine derivative, BN50730, in human plasma and urine by combined liquid chromatography–negative chemical ionization mass spectrometry using a particle beam interface

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Abstract

A new simple and sensitive assay has been developed for the quantitative measurement of BN50730 at the picomole level in human plasma and urine. The drug and the internal standard (BN50765) were measured by combined liquid chromatography–negative chemical ionization mass spectrometry with methane as the reagent gas. A simple solid–liquid extraction procedure was used to isolate BN50730 from complex biological matrices. Mild operating conditions were required to assay the parent drug with a particle beam interface from Hewlett-Packard. The mass spectrometer was tuned to monitor the intense ion *m/z* 333, which was generated in the ion source by a dissociative capture process. This assay was performed with 1 ml of plasma or 0.1 ml of urine, and the quantification limit of the method was statistically calculated as 1 ng ml^{-1}. The very low relative standard deviation and mean percentage of error calculated during the different within-day or between-day repeatability assays clearly demonstrate the ruggedness of the technique for the routine determination of BN50730 in the biological fluids. Some preliminary results on the pharmacokinetics of the drug are presented to illustrate the applicability of this new powerful LC–MS method.

1. Introduction

BN50730 (**I**), [9-(4-methoxyphenylthiocarbamoyl)-6-(2-chlorophenyl)-1-methyl-4, 7, 8, 10-tetrahydro-pyrido[4′,3′-4,5] thieno[3,2-*f*]-1,2,4-triazolo [4,3-*a*]-1, 4-diazepine $C_{26}H_{23}O_1N_6Cl_1S_2$], is a synthetic hetrazepine derivative with potent platelet activating factor (PAF) antagonizing properties (Fig. 1) [1,2].

Compound **I** is a new compound currently under investigation for its antiallergic and antiasthmatic activities. In vivo, **I** is extensively metabolized, and preliminary studies have emphasized the need for a powerful analytical method allowing the quantitative determination of the parent drug at the picomole level [3].

Because hetrazepine derivatives are prone to thermal decomposition, gas chromatographic techniques were not used for this assay.

Liquid chromatography (LC) combined with

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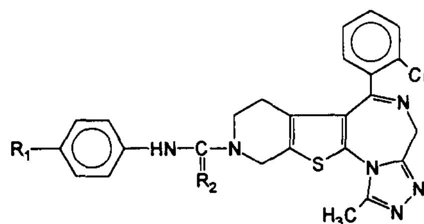

R_1 = OCH_3 R_2 = S BN50730 (I)

R_1 = F R_2 = S BN50765 (II : internal standard)

Fig. 1. Chemical structure of BN50730 and internal standard.

negative chemical ionization mass spectrometry (NCI-MS) offered a more favourable starting point for a specific and sensitive assay of **I** in complex biological matrices down to 1 ng ml^{-1}. We have developed an original LC–NCI-MS technique, with methane as the reagent gas, to measure with precision and accuracy the low levels of **I**. The plasma and urine extracts were analyzed using a particle beam interface, and the detection was carried out by recording the intense and characteristic ion m/z 333 in the selected-ion monitoring (SIM) mode.

This method has been used routinely for more than 16 months in the course of many different pharmacokinetic studies. The feasibility of the technique is exemplified by preliminary results on the quantitative measurement of **I** in the plasma and urine samples of twelve young healthy volunteers following a 90-mg single oral administration of the drug.

2. Experimental

2.1. Chemicals

BN50730 (**I**) and BN50765 (**II**: internal standard) were kindly supplied by the Institut Henri Beaufour (Paris, France). Methyl alcohol for HPLC was purchased from Carlo Erba (Milan, Italy). Water Hypersolv and buffer pH 5 Titrisol were obtained from Merck (Darmstadt, Germany). Acetonitrile was commercially available from SDS (Peypin, France). The Adsorbex C_{18} disposable extraction columns were supplied by Merck. The mobile phase was filtered under vacuum through a 0.20 μm pore size filter purchased from Millipore (Bedford, MA, USA).

In order to avoid any subsequent sample contamination, disposable glass tubes with Teflon caps were used during this assay. Before analysis, all glassware was carefully cleaned and then rinsed with double distilled water.

2.2. Standard curves

Stock solutions of **I** and **II** were prepared by dissolving each pure reference standard in methyl alcohol in order to obtain a 100 ng μl^{-1} primary concentration. These solutions were divided into separate glass vials (1 ml) and stored at −20°C until used.

Working standard solutions were obtained by appropriate dilutions of the stock solutions in methyl alcohol, and were protected from light with aluminium foil and stored at 4°C.

Blood was withdrawn from healthy volunteers into heparinized tubes which were immediately centrifuged at 2600 g for 15 min. Plasma samples were decanted and mixed into separate polyethylene tubes in order to get different sets of drug-free control plasma. Control urine was also collected and stored at −20°C until used.

A ten-point standard curve was prepared daily by spiking aliquots of drug-free human plasma (1 ml) with 40 μl of a 1.25 ng μl^{-1} internal standard solution, and the range of plasma concentrations was 1 to 400 ng ml^{-1}. Urine standard curves were prepared in a similar way: aliquots of blank urine (0.1 ml) were fortified with 50 ng of **II** and various amounts of **I** ranging from 1 to 200 ng.

The blank specimens were prepared by spiking control plasma or urine samples only with the internal standard solution.

2.3. Extraction from plasma and urine samples

Plasma (1 ml) or urine (0.1 ml), fortified as stated above with the BN50765 solution, was placed into a 5-ml glass culture tube. Plasma samples were diluted with 1 ml of water, and urine was buffered with 0.9 ml of buffer pH 5.

After briefly stirring using a vortex mixer, the biological sample was applied to the Adsorbex C_{18} disposable column. The column was washed twice with 1 ml of water, and the compounds of interest were eluted from the column at atmospheric pressure using 1 ml of pure methyl alcohol. The solvent was collected into a 10-ml glass screw-capped tube, and evaporated to dryness at 45°C under a gentle stream of nitrogen. The residue was dissolved in 150 μl of mobile phase, and a 40-μl sampling volume was injected into the HPLC–MS system.

2.4. HPLC–MS analysis

Liquid chromatography was performed with a Hewlett-Packard HP1050 quaternary pump and a Merck AS2000 automatic sampler equipped with a 100-μl Rheodyne loop. The chromatographic column (100 mm × 4.6 mm I.D) was packed with a 3-μm particle size Microspher C_{18} stationary phase from Chrompack (Les Ulis, France), and was protected with a guard column (20 mm × 2 mm I.D) filled with a pellicular support. The elution was carried out with a mixture of acetonitrile–water (40:60, v/v) at a 0.40 ml min^{-1} flow rate.

The HPLC system was connected to a Hewlett-Packard HP59980A particle beam interface. The LC effluent was converted into an aerosol in a pneumatic nebulizer, where helium was introduced coaxially at a flow rate of ca. 1.5 l min^{-1}. The aerosol sprayed into a desolvation chamber in which the temperature was held at 50°C. Finally, a beam of virtually solvent-free particles was introduced into a Hewlett-Packard HP5988A mass spectrometer via a transfer tube.

The MS system was operated in the NCI mode with an electron energy of 250 eV, an emission current of 300 μA, and an ion source temperature of 190°C.

Prior to the analysis, the instrument was tuned using the negative ions m/z 414, 595 and 633 from the perfluorotributylamine (PFTBA) calibrant gas.

The methane reagent gas was admitted via a gas-flow controller to an indicated ion source pressure of 0.9 Torr (1 Torr = 133.322 Pa). The NCI mass spectra of **I** and **II** (100 ng of each analyte injected into the column) were recorded during a HPLC run by scanning the quadrupole mass filter every 1.2 s from m/z 120 to m/z 600. The mass peak width was set at 0.55 u over the whole mass range.

Quantitative determination of the drug was performed by focusing the instrument in the selected-ion monitoring mode in order to measure the ion m/z 333 shared by the two compounds **I** and **II**. The HP 5988A mass spectrometer was interfaced with a Pascal data operating system. The raw data analysis was performed on dedicated computers with customized software developed in-house to meet specific requirements. These programs have previously been validated in accordance with the Good Laboratory Practices (GLP) recommendations. The data acquisition computer and the workstations were linked to a Hewlett-Packard Chemlan local area network.

2.5. Validation procedure

To assess the within-day precision and accuracy of the method, repeatability assays were carried out at five different concentrations (1, 5, 40, 100 and 200 ng ml^{-1}). The spiked plasma specimens were analyzed by the same operator, and for each concentration level, the relative standard deviation and mean percentage of error were calculated.

The limit of quantification (LOQ), that must be determined with a stated confidence level, was defined as the lowest detectable concentration yielding a signal significantly higher than the mean signal measured in representative control specimens [4–7]. First, to calculate the LOQ of the method, a repeatability assay was performed with eight drug-free blank plasma samples. The mean signal (Y_{bl}) observed at the retention time of **I** and the associated standard deviation (S_{bl}) were used to calculate a theoretical value (Y_{th}) of the quantification limit [8].

Then, a repeatability assay was carried out with eight replicate plasma samples spiked with a BN50730 concentration equal to this theoretical limit of quantification.

The mean signal value (Y_{LOQ}) was statistically compared to the mean signal (Y_{bl}) obtained with the control specimens. After testing variance homogeneity ($p < 0.05$), a Student t-test or a Welch test was used in order to demonstrate that Y_{LOQ} was significantly higher than Y_{bl} at the 97.5% probability level [9,10].

In addition, the effects of freeze–thaw cycles were investigated in plasma specimens fortified with 6.5 and 121.8 ng ml^{-1} of **I**. The stability of the drug was also tested at two different levels in urine (6.1 and 122.8 ng ml^{-1}) and blood (6.4 and 104.9 ng ml^{-1}) samples.

2.6. *Quality controls*

Throughout the study, quality control (QC) samples were analyzed to evaluate the between-day precision and accuracy of the technique. The QC samples were prepared blind to the analyst by adding known amounts of **I** to blank samples. The working standard solutions used to spike the control specimens were different from the ones used to prepare the standard curves. The QC samples were stored at −20°C until analysis, and a set of 3 to 6 QC samples was daily assayed together with the "real-life" biological samples collected during the pharmacokinetic studies. After decoding the results, the lack of any systematic bias in the assay of the drug was checked. For that reason, a regression analysis was performed by plotting the found values vs. the theoretical ones. The slope and the intercept were statistically compared to the theoretical values (1 and 0, respectively) using a Student t-test.

2.7. *Drug administration*

Twelve healthy male subjects completed the pharmacokinetic study. They were examined to be in good health through their medical history, physical examination and a routine laboratory test. Each subject received a single 90-mg oral dose of BN50730 as tablets. Blood samples were withdrawn in heparinized tubes at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 12, 18 and 24 h following the administration of the drug. Plasma was separated from the red blood cells by centrifugation at 4°C (2400 g for 15 min) and the samples were stored deep frozen at −20°C for subsequent HPLC–MS analysis.

Urine samples were collected in polyethylene flasks at the following time intervals: 0–2 h, 2–6 h, 6–12 h and 12–24 h. Volumes were carefully measured and recorded. Aliquots of 10 ml were transferred into labeled tubes, and maintained at −20°C until they were assayed.

3. Results and discussion

3.1. *Sample pretreatment and analysis*

Prior to this work, we developed a simple HPLC method with ultraviolet detection suitable for evaluating the chemical behaviour of **I** during the extraction process. These investigations have clearly shown that the solid-phase extraction technique, using the Adsorbex C_{18} disposable columns, was more efficient than a liquid–liquid extraction procedure. Moreover, some organic solvents, such as ethyl acetate, were prohibited because they were at the origin of a sample degradation leading to the formation of the NHPTT product in the biological extracts (Fig. 2).

The rapid extraction process described in this paper provides a clean residue devoid of most of the impurities, so that the total chromatographic run time is reduced to less than 10 min. Under these conditions, the laboratory throughput was increased to 50 biological samples per day using an automatic injector.

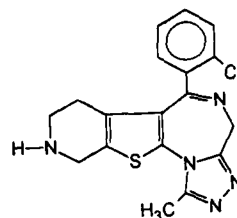

Fig. 2. Chemical structure of the NHPTT degradation product.

3.2. HPLC–MS analysis

To optimize the chromatographic parameters, different stationary and mobile phases were tested, taking into account that the particle beam interface can operate at flow rates as high as 1 ml min^{-1}; the optimum is at ca. 0.45 ml min^{-1}. As a result, a short column packed with a C_{18} stationary phase was found to be suitable for this assay. The two analytes were well resolved, without any broadening, and in our analytical conditions, the retention times of **I** and **II** were 7.0 min and 8.4 min, respectively. The two chromatographic peaks approximated a Gaussian shape curve, and the base peak width of each analyte was less than 45 s. The peak width calculated at half height was about 14 s. A dwell time of 200 ms per mass range led to a minimum of 46 data points for each compound measurement, taking into account the total system overhead time.

First- and second-stage skimmers in the momentum separator are subject to occasional clogging when buffers are added into the mobile phase. In this experiment, the simple acetonitrile–water mixture used as the mobile phase overcomes this major problem and the particle beam interface has been running routinely over long periods of time (>3 months) without any maintenance.

Under mild analysis conditions, the NCI mass spectrum of **I**, obtained with methane as the reactant gas, exhibits characteristic ions in the middle mass region as shown in Fig. 3. The molecular anion m/z 534 is absent because this ion subsequently undergoes the loss of HCl (m/z 498 with 0.5% relative intensity) and then the 4-methoxy phenyl thiocarbamoyl moiety, leading to the formation of the base peak measured at m/z 333.

The mass spectrum of the internal standard is similar to that of **I** due to the successive loss of HCl and the 4-fluorophenylthiocarbamoyl fragment from the substituted hetrazepine structure.

3.3. Recovery

Suitable amounts of the pure reference standards were injected into the liquid chromatographic system, and the peak areas were compared with the ones obtained after the injection of control plasma extracts spiked with the same concentrations of **I**. The overall recovery was ca. 90% for both analytes.

3.4. Standard curves

The daily plasma calibration curves, obtained throughout this study by plotting the peak area ratios m/z 333 *vs.* the known concentrations of **I**, were straight lines over the concentration range 1 to 400 ng ml^{-1}. The non-weighted least-squares regression analysis crossed near the origin with an intercept value close to zero. The between-day linearity and reproducibility were evaluated using the regression parameters of 12 calibration curves elaborated and run over a two-week period by different analysts. The slopes were consistent from day to day (R.S.D. = 5.5%), and there was a minimal scattering of the ten calibration points all along the regression lines. The current plasma standard curve range was suitable in most pharmacokinetic studies, but was of course adjusted depending on the dose administered to humans.

3.5. Precision and accuracy

All sources of variability were under control due to the use of an internal standard with a chemical structure quite close to that of **I**. The relative standard deviations of the different plasma repeatability assays calculated the same day by the same analyst were less than 5.0%, and the mean percentages of error ranged from −6.26 to 3.81% (Table 1).

The ruggedness of the technique was tested by analyzing different sets of plasma QC samples over a very long period of time (more than 16 months). The results of the between-day precision and accuracy were quite similar to those obtained during the intra-day assay: the R.S.D. values were between 4.92 and 6.19% and mean percentages of error ranged from −3.64 to 1.35% for BN50730 concentrations tested from 2.5 to 150 ng ml^{-1} (Table 2).

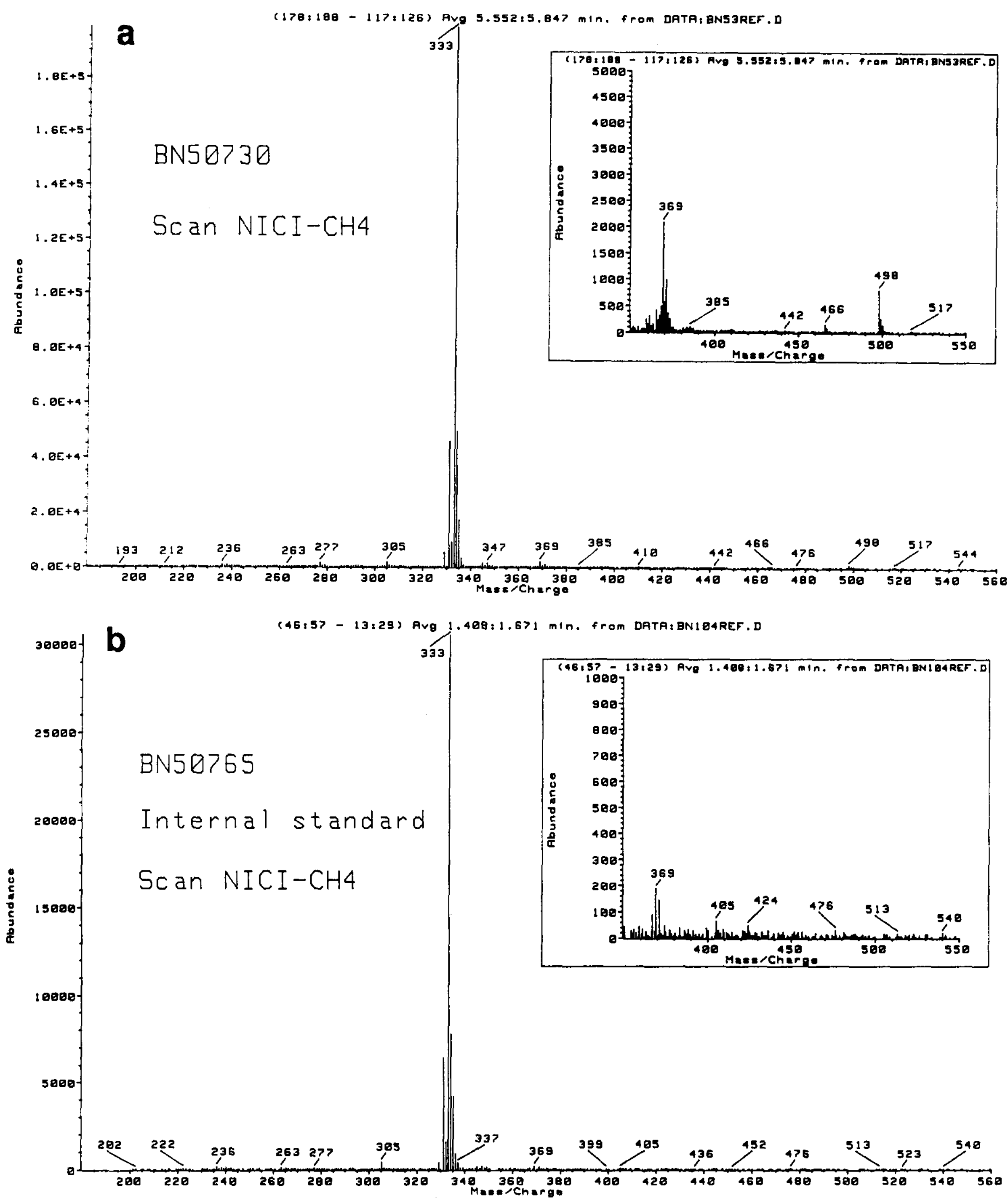

Fig. 3. Methane negative chemical ionization mass spectra of (a) pure BN50730 and (b) BN50765.

Table 1
Intra-day precision and accuracy of the HPLC–MS method in the plasma samples

Theoretical concentration (ng/ml)	n	Mean found concentration (ng/ml)	S.D.	R.S.D. (%)	Mean error (%)
1	8	1.013	0.031	3.12	1.33
5	6	4.686	0.113	2.41	−6.26
40	6	39.254	1.923	4.90	−1.87
100	6	103.809	2.907	2.80	3.81
200	8	197.246	5.450	2.76	−1.38

3.6. Limit of quantification

The theoretical LOQ, based upon the results obtained after the HPLC–MS analysis of eight different control specimens, was calculated to be equal to $Y_{th} = 0.1$ ng ml^{-1}. For feasibility reasons, and bearing in mind that the method must be used for a very long period of time, this theoretical limit of quantification could obviously not be retained, and therefore a repeatability assay was performed at the 1 ng ml^{-1} level.

Because of variance homogeneity ($F_{cal} = 2.79 < F_{tab} = 4.43$, $p < 0.05$), a Student t-test was applied to demonstrate that the mean signal value $Y_{LOQ} \pm$ S.D. ($3.201 \cdot 10^{-2} \pm 1.081 \cdot 10^{-3}$) calculated from the LOQ repeatability assay was significantly different from the one (Y_{bl}) observed with the eight blank samples ($9.739 \cdot 10^{-4} \pm 1.805 \cdot 10^{-3}$).

The $t_{cal} = -41.7$ was much higher than the $t_{tab} = 4.14$ at the 0.1% probability level for $n =$ 14 degrees of freedom ($p < 0.001$). Owing to the low R.S.D. (3.12%) and mean percentage error (1.33%), this concentration level was validated as the quantification limit of the method (Table 3).

A typical raw mass chromatogram recorded after the HPLC–MS analysis of a control plasma sample is shown in Fig. 4. Control specimens spiked with 1 ng ml^{-1} (LOQ) and 20 ng ml^{-1} of **I** are presented in Fig. 5.

When a plasma extract corresponding to the LOQ of the method was analyzed, the signal measured at the retention time of **I** was equivalent to about 235 picograms (ca. 0.45 picomole of **I**) injected into the HPLC–MS system.

3.7. Quality control analysis

QC analysis ($n = 518$) has shown that the found plasma concentrations were correlated

Table 2
Between-day precision and accuracy of the HPLC–MS method calculated over a 16-month period with 518 QC plasma samples

Theoretical concentration (ng/ml)	n	Mean found concentration (ng/ml)	S.D.	R.S.D. (%)	Mean error (%)
2.5	127	2.489	0.154	6.19	−0.43
5	70	4.919	0.247	5.01	−1.63
10	10	10.135	0.499	4.92	1.35
20	147	20.179	1.155	5.72	0.89
40	123	40.113	2.224	5.54	0.28
50	8	49.756	3.061	6.15	−0.49
100	26	97.293	4.932	5.07	−2.71
150	7	144.537	7.523	5.21	−3.64

Table 3
Validation procedure for the determination of the quantification limit (LOQ)

Sample number	Peak area ratios (m/z 333)		Back-calculated concentration (ng ml^{-1})
	Blank samples	LOQ samples	LOQ samples
1	0.000000	0.031767	1.006
2	0.000000	0.032636	1.032
3	0.004023	0.031235	0.990
4	0.000000	0.030322	0.964
5	0.003768	0.032065	1.015
6	0.000000	0.031613	1.002
7	0.000000	0.032402	1.025
8	0.000000	0.034002	1.071
Mean	$Y_{bl} = 9.739 \cdot 10^{-4}$	$Y_{LOQ} = 3.201 \cdot 10^{-2}$	1.013
S.D.	$1.805 \cdot 10^{-3}$	$1.081 \cdot 10^{-3}$	0.0316
n	8	8	8
R.S.D. (%)		3.38	3.12
Variance	$3.250 \cdot 10^{-6}$	$1.160 \cdot 10^{-6}$	

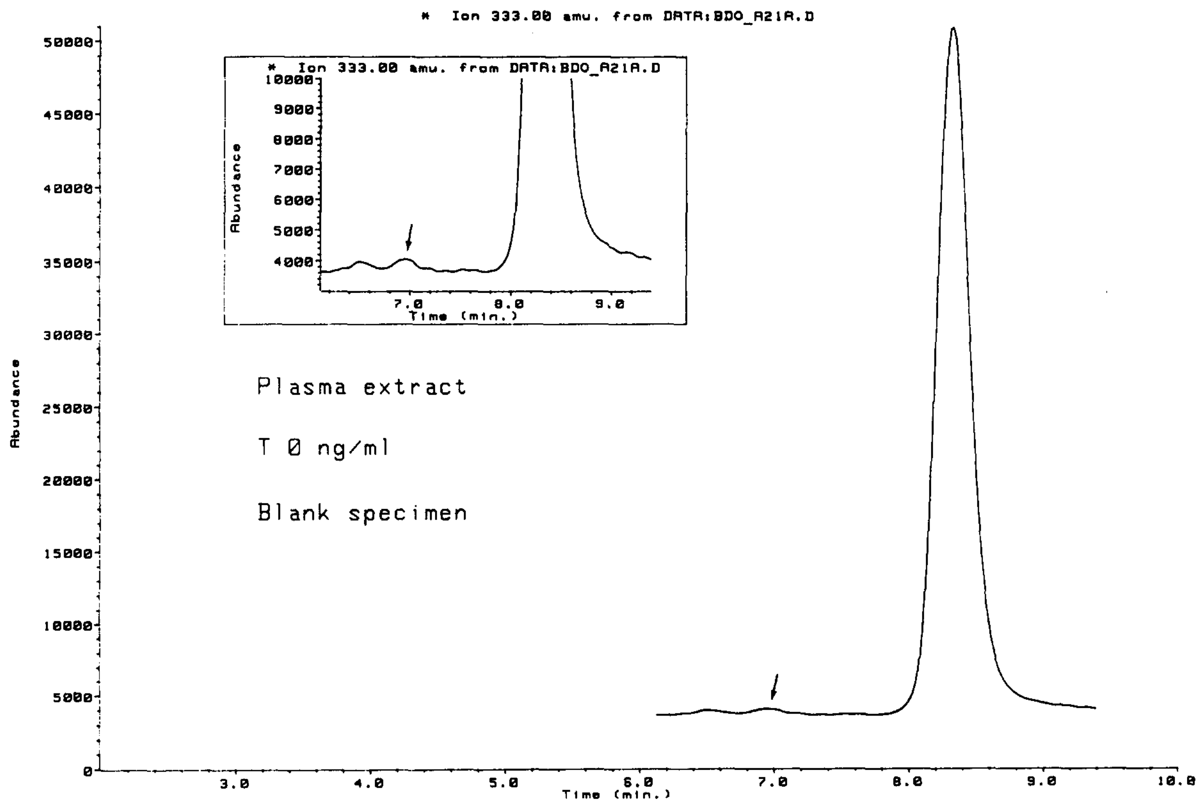

Fig. 4. Typical mass chromatogram obtained from a blank plasma sample spiked with 50 ng of BN50765 (arrow indicates the retention time of BN50730).

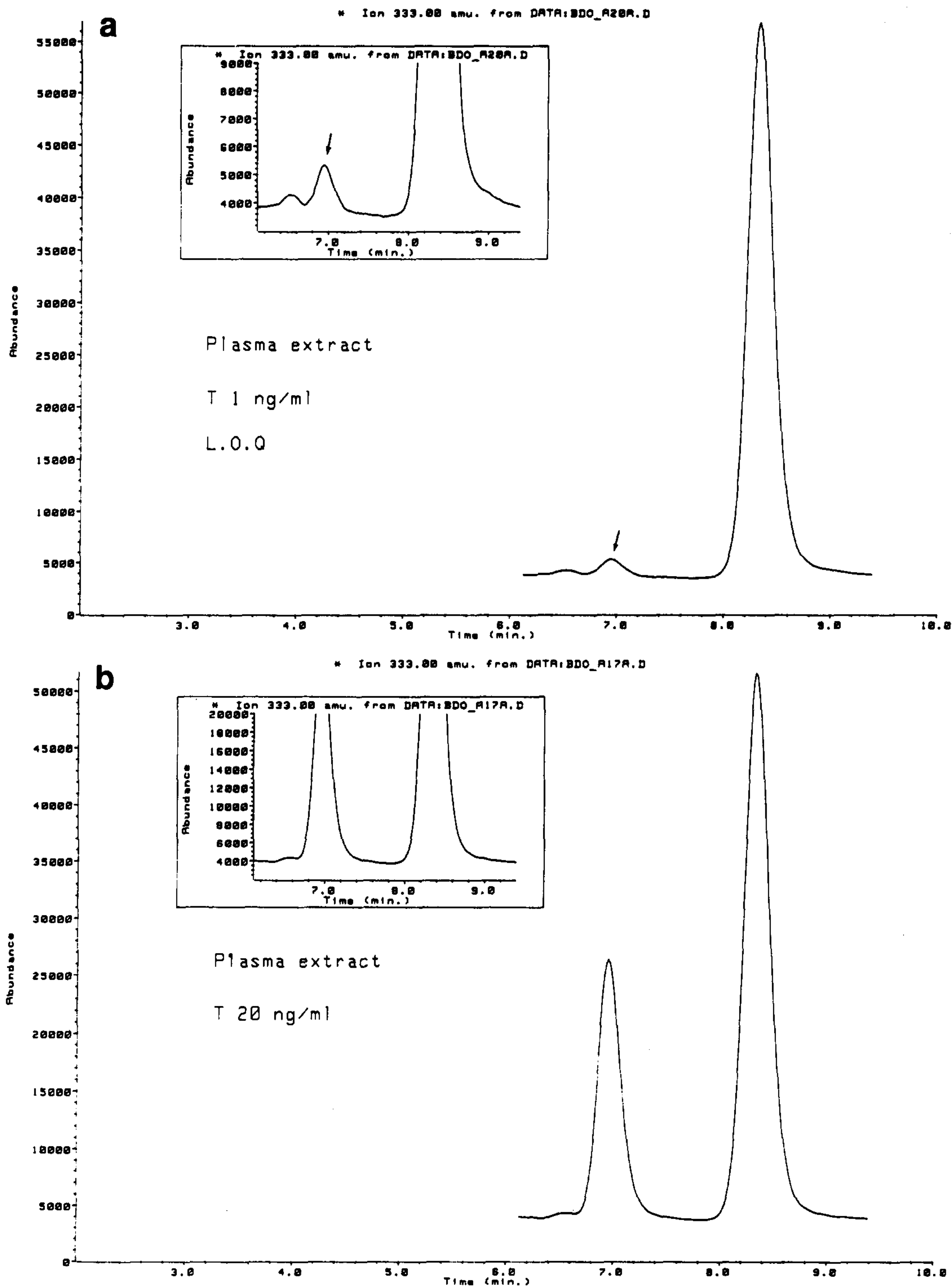

Fig. 5. Selected-ion monitoring traces recorded during the HPLC–MS analysis of a plasma sample fortified with (a) 1 ng ml^{-1} (LOQ) and (b) 20 ng ml^{-1} of BN50730.

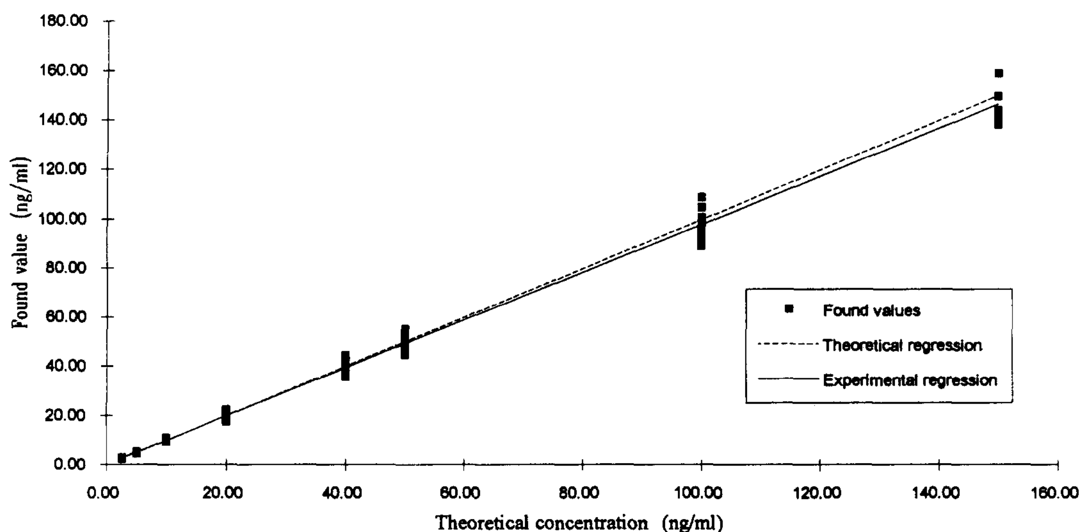

Fig. 6. Regression analysis of the 518 plasma quality control samples.

($r = 0.997338$) with the theoretical ones as shown in Fig. 6.

The $t_{intercept} = 4.200$ and $t_{slope} = -8.210$ values were higher than the limit ($t_{tab} = 1.960$) of the t-table: thus, the intercept ± S.D. (0.483014 ± 0.114999) and slope (0.974254 ± 0.003136) values were slightly different from zero and one at the 95% probability level for $n-2$ degrees of freedom. However, this very low bias (−2.57%) has no analytical significance, taking into account

Table 4
Experimental design of the BN50730 stability test in blood, plasma and urine samples

Step	Blood samples		Plasma samples		Urine samples	
	Series B1	Series B2	Series P1	Series P2	Series U1	Series U2
1	Two series of 5 blood samples (6.4 and 104.9 ng ml^{-1})		Two series of 5 plasma samples (6.5 and 121.8 ng ml^{-1})		Two series of 5 urine samples (6.2 and 122.8 ng ml^{-1})	
2	Stored 3 h at ambient temp.	Stored 6 h at ambient temp.	Stored 1 h at ambient temp.	Stored 1 h at ambient temp.	Stored 36 h at ambient temp.	Stored 36 h at ambient temp.
3	Frozen (−20°C) for 1 month	Frozen (−20°C) for 1 month	Frozen (−20°C) for 48 h	Frozen (−20°C) for 1 month	Frozen (−20°C) for 48 h	Frozen (−20°C) for 1 month
4	Thawed for the HPLC–MS run	Thawed for the HPLC–MS run	Thawed and stored 24 h at ambient temp.	Thawed for the HPLC–MS run	Thawed and stored 24 h at ambient temp.	Thawed for the HPLC–MS run
5			Frozen (−20°C) for 1 month		Frozen (−20°C) for 1 month	
6			Thawed for the HPLC–MS run		Thawed for the HPLC–MS run	

that the QC samples were analyzed over a very long period of time (16 months) by six different technicians. Moreover, when the QC regression curve is split between the low (2.5 to 20 ng ml^{-1}) and high concentration (20 to 150 ng ml^{-1}) levels, this apparent systematic error is totally absent.

3.8. Stability of the biological samples

The stability of **I** was tested in different biological matrices stored at room temperature and −20°C prior to the HPLC–MS analysis. Two sets ($n = 5$) of blood, plasma and urine samples were spiked with known amounts of **I** and then subjected to various cycles of freezing and thawing. The experimental design of the stability test is summarized in the Table 4.

These investigations have shown that no major difference was observed in the mean calculated concentrations of the spiked blood, plasma and urine samples compared to each theoretical value (Table 4). However, in order no to damage the biological specimens collected during the pharmacokinetic or clinical studies, unexpected thawing must be avoided once the samples are frozen at −20°C.

3.9. Pharmacokinetic data

The applicability of this technique was exemplified by the results of the BN50730 plasma and urine concentrations measured after the oral administration of a single 90-mg dose to twelve subjects. Pharmacokinetic analysis have shown that the C_{max} value (mean ± s.e.m.) was equal to 94.75 ± 18.86 ng ml^{-1} and most of the drug was absorbed within 2.5 h ($t_{max} = 2.05 \pm 0.54$ h). The elimination rate constant (λ_z) and the half-life

Table 5
Results of the stability test in the blood, plasma and urine samples

Blood samples	B1	B2	B1	B2
Theoretical concentration (ng ml^{-1})	6.4		104.9	
Mean measured concentration ($n = 5$)	6.21	6.61	101.40	104.55
S.D.	0.823	0.302	2.651	3.402
R.S.D. (%)	13.27	4.57	2.61	3.25
Plasma samples	**P1**	**P2**	**P1**	**P2**
Theoretical concentration (ng ml^{-1})	6.5		121.8	
Mean measured concentration ($n = 5$)	6.62	6.64	123.89	132.69
S.D.	0.161	0.200	2.223	2.245
R.S.D. (%)	2.44	3.01	1.79	1.69
Urine samples	**U1**	**U2**	**U1**	**U2**
Theoretical concentration (ng ml^{-1})	6.2		122.8	
Mean measured concentration ($n = 5$)	5.94	6.69	116.89	116.23
S.D.	0.363	0.382	3.196	4.624
R.S.D. (%)	6.11	5.71	2.73	3.98

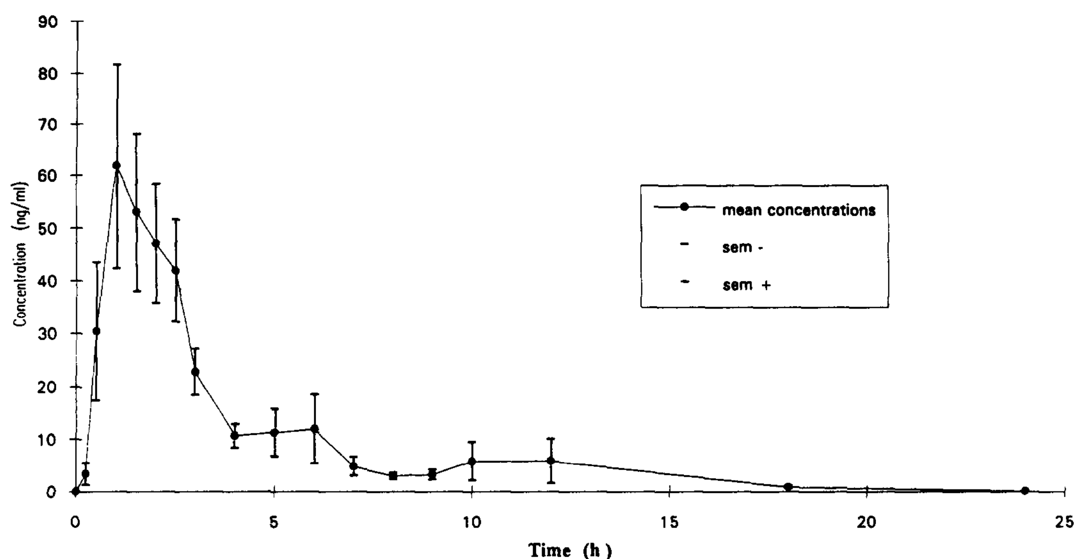

Fig. 7. Mean plasma concentrations ± S.E.M. of BN50730 vs. time following a single 90-mg oral administration of the drug to twelve healthy subjects.

value $t_{1/2z}$(8.01 ± 1.97 h) were calculated by linear least-squares fit of the data points in terminal slope (time vs. log concentrations). The arithmetic plot of the mean plasma concentrations ± s.e.m. ($n = 12$) vs. time is presented in Fig. 7. The total amount ± s.e.m. of **I** measured in the 0–24 h urinary fraction was 28.41 ± 5.57 μg. Therefore, the drug was extensively metabolized and less than 0.35‰ of the parent compound was excreted in urine as the unchanged form.

4. Conclusion

The pharmacokinetic handling of **I** can only be achieved if accurate and precise raw analytical data are obtained. The method used to quantify this drug in the biological samples should be carefully optimized so that the reliability of the procedure greatly depends on the criteria used to validate the assay. The particle beam HPLC–MS assay described in this paper affords a highly sensitive and specific technique for the determination of **I** at the picomole level in plasma and urine samples. For more than three years, the Hewlett-Packard 59980 particle beam interface was successfully used in our laboratories for the development of new analytical methods. This interface was found to be particularly well adapted for routine quantitative determination of thermolabile compounds present at the nanogram to sub-nanogram levels in complex biological matrices [11–14]. During this assay, a very intense signal was generated in the ion source, and when the m/z 333 ion was monitored under NCI conditions with methane as the reactant gas, the quantification limit was calculated as 1 ng ml^{-1}. The low relative standard deviation and mean percentage error values calculated during the different repeatability assays have demonstrated that, even around the quantification limit, the precision and accuracy of the method were suitable for routine analysis of BN50730. These results were confirmed by the ones obtained after the HPLC–MS measurement of 518 QC specimens analyzed blind to the analyst. This method has been successfully applied to a large batch of samples ($\geqslant$9000) assayed over a 16-month period and was routinely carried out for pharmacokinetic, clinical and toxicological studies.

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