Pergamon

0731-7085(95)01290-7

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 4/5, pp. 607–614, 1995
Elsevier Science Ltd
Printed in Great Britain
0731–7085/95 $9.50 + 0.00

Qualitative analysis of potential metabolites and degradation products of a new antiinfective drug in rat urine, using HPLC with radiochemical detection and HPLC–mass spectrometry*

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Abstract: High-performance liquid chromatography (HPLC) was used in combination with radioactivity detection and mass spectrometry (MS) to elucidate the metabolic fate of GV104326, a novel tricyclic β-lactam antibacterial agent. Metabolic profiles were obtained by analysis of rat urine samples collected after intravenous administration of the ^{14}C-labelled drug at the dose of 50 mg kg^{-1} (12.95 MBq kg^{-1}). Methods for solid-phase extraction from urine samples and for reversed-phase chromatographic separation of drug related material were developed. HPLC–MS was used to confirm that the parent compound corresponded to the principal peak in the chromatograms, and two minor peaks were identified as potential metabolites of GV104326. They were shown to be an open β-lactam ring derivative (GV173923) and a dimeric compound (GV196359).

Keywords: *Rat; intravenous administration; radioactivity; urinary excretion; metabolic profile, strong-anion exchange; HPLC; urine assay; solid-phase extraction; particle-beam/chemical ionization MS.*

Introduction

GV104326 is a member of a new class of tricyclic beta-lactam antibacterial agents [1]. The structure of the ^{14}C labelled compound used in this work is shown in Fig. 1.

It has been shown for other compounds with β-lactam structures, like the penem compound SUN 5555 [2], that chemical and enzymatic degradation led to opening of the β-lactam ring and that the principal degradation product corresponded to the main metabolite.

Previous studies involving intravenous administration of ^{14}C-GV104326 to rats and dogs showed that the majority of the administered radioactivity was excreted into urine (urinary recovery of radioactivity: 76% for rat, 90% for dog).

Hydrolysis of the β-lactam ring is a well known reaction observed for penicillins [3] and penem compounds [4–8]. Therefore, GV173923 (Fig. 2), which is the degradation product of GV104326 formed by cleavage of

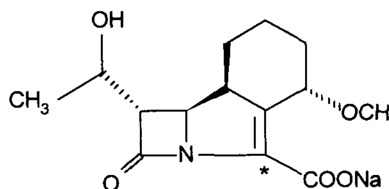

Figure 1
Structure of radiolabelled GV104326 (* = position of ^{14}C).

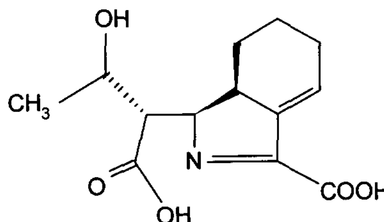

Figure 2
Structure of GV173923 (degradation product of GV104326).

*Presented at the Fifth International Symposium on Pharmaceutical and Biomedical Analysis, Stockholm, Sweden, September 1994.

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the β-lactam ring, was used as a reference compound for the development of chromatographic methods.

A method for the assay of GV104326 in human plasma has already been reported [9]. This paper describes how solid-phase extraction, HPLC and particle-beam mass spectrometry have been used to investigate the metabolites of GV104326 in rat urine.

Results and Discussion

Solid-phase extraction

Three commercially available solid adsorbent materials were evaluated for GV104326 extraction from rat urine. C18 ENVI cartridges were recommended for extracting organic contaminants from water samples. HISEP cartridges were suggested for the separation of small analytes from biological matrices; they contain a hydrophobic phase protected by a hydrophilic network. SAX (strong-anion exchange) cartridges contain a trimethyl quaternary ammonium phase and have been used successfully for GV104326 extraction from human plasma samples [9].

Preliminary screening of these cartridges was performed with 50 μl of an aqueous solution of ^{14}C-GV104326 (10 μg ml^{-1}), washing with 1 ml of water. All experiments were performed in duplicate. Fractions of eluate were collected and the radioactivity counted (Table 1). Both C18 ENVI and SAX cartridges gave good retention of the radiolabel. The same procedure was repeated with 50 μl of rat urine spiked with the radiolabelled drug (10 μg ml^{-1}). The results (Table 1) showed that GV104326 was extracted quantitatively from rat urine only by SAX cartridges. This indicated that a strong polar interaction with the packing was needed for retention of the drug in presence of the matrix.

Urine spiked with 2 mg ml^{-1} ^{14}C-GV104326 were diluted 1:10 with water, then aliquots of increasing volumes were tested using SAX cartridges. All the experiments were performed in duplicate. The resulting recovery values are summarized in Table 2. A sample volume of 5 ml (500 μl of urine) was shown to give an acceptable recovery.

GV104326 was eluted from SPE cartridges with a solution of citrate buffer (0.5 M at pH 7.9). The highest recovery was obtained when using two aliquots of 0.5 ml. No significant increase in recovery was noted after elution with two further aliquots of 0.5 ml of citrate.

Equipment

The HPLC system consisted of a model 600E solvent delivery pump (Waters), a UV detector model 870 (Jasco) and a WISP autosampler (Waters). A model 'Flo-One' Beta detector (Radiomatic) equipped with a 0.5 ml liquid scintillation cell was used for on-line radioactivity detection.

The concentrations of radioactivity in the samples were determined using a liquid scintillation counter, model MINAXI-TRICARB 4000 (Packard) correcting for quenching by the external standardization technique (SIE).

For HPLC–MS analysis a model HP 1050 solvent delivery system and UV-detector

Table 1
Percentage of ^{14}C-GV104326 not retained by different SPE cartridges

Sample	C18ENVI	HISEP	SAX
0.5 μg ^{14}C-GV104326 in water	1.3	15.1	1.1
0.5 μg ^{14}C-GV104326 in rat urine	29.9	58.0	0.9

Table 2
Solid-phase extraction of ^{14}C-GV104326 from urine by SAX cartridges: percentage of radiolabel recovered for different volumes of sample

Sample volume (ml)	Amount of GV104326 (μg)	Sample loading %	Cartridge washing %	Cartridges elution %
0.5	100	0.7	1.0	81.0
1.0	200	1.0	0.6	84.0
2.0	400	1.3	0.4	86.0
3.0	600	1.6	0.6	88.0
4.0	800	2.6	1.2	89.0
5.0	1000	5.0	2.3	83.0

(Hewlett–Packard) was coupled with a model HP 5987A single-quadrupole mass spectrometer (Hewlett–Packard) through a model 59980A Particle Beam (PB) interface (Hewlett–Packard). A model M45 solvent delivery pump (Waters) was used for post-column solvent addition.

Animal experiments

Six male Wistar rats (Charles River, Como, Italy) were treated once by intravenous administration in the caudal vein of ^{14}C-GV104326 at the dose of 50 mg kg^{-1} (12.95MBq kg^{-1}) as the pure free acid. The dosing solution was prepared by dissolving in water for injection the ^{14}C-labelled and cold GV104326, to give a concentration of 25 mg ml^{-1} as total free acid; this was then administered at a dose volume of 2 ml kg^{-1}. The animals were weighed and identified before dosage. Then they were transferred into plastic metabolic cages. Urine samples were collected in tubes kept cool by dry-ice over 0–6 h intervals after administration, then stored at −80°C.

Experimental

Materials and reagents

GV104326 was supplied by Chemical Development, Glaxo S.p.A. (Verona, Italy) GV173923 and GV196359 were provided by the Analytical Department, Glaxo S.p.A. Verona, Italy. ^{14}C-GV104326 was synthesized by the Isotope Chemistry Department, Glaxo Research and Development (Greenford, UK). Its specific activity was: 1.51 MBq mg^{-1} (40.8 μCi mg^{-1}) as the sodium salt and 1.63 MBq mg^{-1} (44.05 μCi mg^{-1}) as the free acid.

Liquid scintillation fluid (Picofluor 40) was supplied by Packard Inst. (Pero, Milano). Acetonitrile (HPLC grade) was obtained from Carlo Erba (Milan, Italy). Ammonium acetate and tri-sodium citrate 2-hydrate (analytical grade) were supplied by Bracco (Milan, Italy). Water was purified through a Milli RO/Milli Q system (Millipore, Milan, Italy).

The following solid-phase extraction (SPE) cartridges were used: C18 ENVI (500 mg, Supelco, PA, USA); HISEP (500 mg, Supelco, PA, USA); SAX (500 mg, Varian, CA, USA). All SPE packings were conditioned according to the recommendations of the suppliers.

HPLC analysis was carried out with a 250 × 4.6 mm column, packed with Spherisorb ODS-2, 5 μm (GSG, Milan, Italy). A guard-column 2 × 0.4 cm, packed with 40-μm Lichrosorb, was used.

HPLC–MS analysis was performed with a narrow-bore column (250 × 2 mm) packed with the same stationary phase.

In conclusion, the purification of the urine samples was performed by SPE with SAX Bond–Elut cartridges (500 mg) according to the following procedure: (1) 0.5 ml of urine was diluted to 5 ml with water; (2) diluted urine samples were transferred into extraction cartridges previously activated with 6 ml ammonium acetate (100 mM) and 6 ml water; (3) cartridges were washed with 1 ml water; (4) cartridges were eluted with two consecutive aliquots of 0.5 ml sodium citrate buffer (0.5 M; pH 7.9).

The final SPE conditions were also verified for the extraction of GV173923, the degradation product of GV104326. A urine sample spiked with partially degraded ^{14}C-GV104326 was extracted according to this method. Sample recovery was similar.

Analysis by HPLC with radioactivity detection was performed on the eluate fraction of the ^{14}C-GV104326 degraded solution. Good recovery was also observed for GV173923. No further degradation was observed during extraction either of GV104326 or GV173923.

HPLC conditions

The chromatographic conditions are as reported in Table 3. The UV chromatogram showing GV104326 and GV173923 peak pro-

Table 3
Chromatographic conditions for the analysis of GV104326 and its metabolites in rat urine samples

Time (min)	CH_3CN (%)	NH_4OAc (%) (200 mM)	Flow (ml min^{-1})
0	—	100	1
3.5	—	100	1
10	10	90	1
20	10	90	1
22*	100	—	2
27*	100	—	2
27.5*	—	100	2
32*	—	100	2
33*	—	100	1

*The gradient from 22 to 33 min is for column regeneration.

Guard-column: 40-μm Lichrosorb, 2 × 0.4 cm; column: Spherisorb ODS 2, 5 μm, 25 × 0.46 cm; temperature: ambient; detectors: UV: at 268 nm; radioactivity: ^{14}C; retention times: 12 min (GV173293) and 20 min (GV104326).

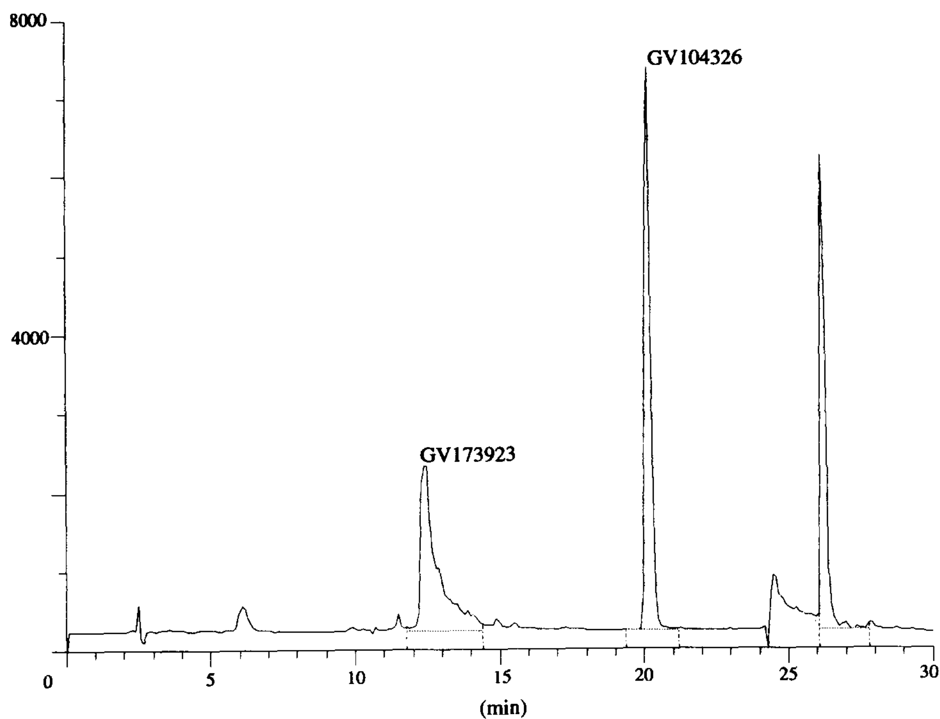

Figure 3
UV chromatographic profile (λ = 268 nm; arbitrary detection units) of standard solution containing (GV173923 (69 μg ml^{-1}) and GV104326 (68 μg ml^{-1}) 50 μl injected. The peaks eluting after 24 min are due to column wash/regeneration.

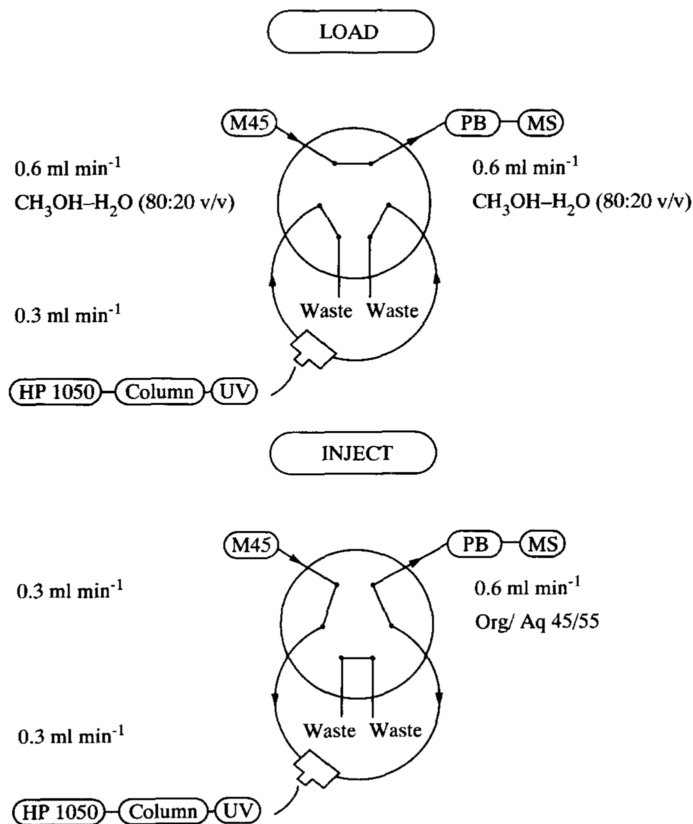

Figure 4
HPLC–PB–MS system configuration for post-column addition of CH_3OH and chromatogram "heart-cutting".

files is shown in Fig. 3. It will be noted that a low acetonitrile concentration is used for chromatographic elution, followed by column cleaning (100% acetonitrile) and regeneration.

HPLC/PB/MS conditions

To optimize the nebulization of the HPLC eluent in the PB interface, the organic solvent concentration in the mobile phase was increased by post-column addition of methanol–water (80:20, v/v) at a flow rate of 0.3 ml min^{-1}. Figure 4 shows the scheme of the system adopted. When the switching valve is in the LOAD position, the PB interface is conditioned with a 0.6 ml min^{-1} flow of CH_3OH–H_2O. In the mean time, the narrow-bore column is eluted at a flow rate of 0.3 ml min^{-1}. "Heart-cutting" of the chromatogram is performed to transfer into the mass spectrometer only the peaks of interest, i.e. those eluting between 4 and 28 min, by switching the valve to the INJECT position. The final eluate enters the PB–MS at a flow rate of 0.6 ml min^{-1}.

The PB interface was operated with the desolvation chamber temperature set at 80°C, the second momentum separator pressure at 0.6 Torr and the helium inlet pressure at 30 psi.

For Chemical Ionization (CI) ammonia was used as reagent gas and the measurements were performed under negative ion conditions. The MS source was kept at 250°C. Full scan acquisition was carried out over the 150–700 Da mass range. Total ion current chromatograms were generated using this range.

Metabolic profiles

Aliquots of 0.5 ml of urine collected after intravenous administration of ^{14}C-GV104326 to rats, were extracted by SAX cartridges and aliquots of 50 μl of the extracts were injected into the liquid chromatographic system. UV and radioactivity chromatograms were obtained. A typical chromatographic profile for a rat urine sample is reported in Fig. 5.

The main peak in all the chromatograms was shown to be GV104326; a peak at the same retention time as GV173923 was observed and

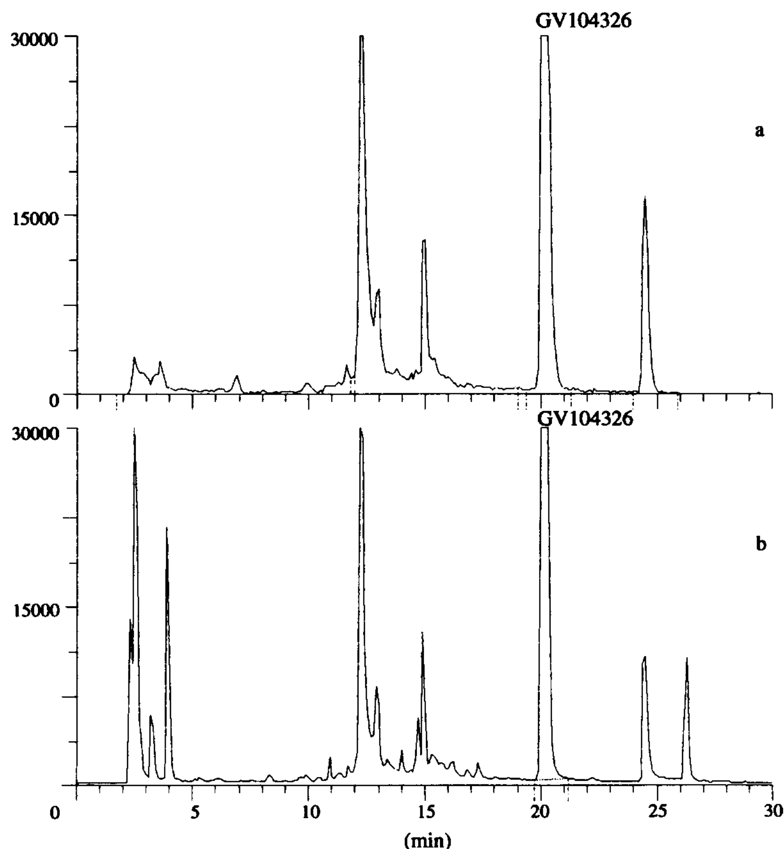

Figure 5
Metabolic profiles of GV104326 from 0–6 h; urine collected from a male rat after intravenous administration of ^{14}C-labelled drug at the dose of 50 mg kg^{-1}: (a) radioactivity (^{14}C) detection (DPM units). (b) UV absorption (λ = 268 nm) detection (arbitrary units).

its identity verified by adding GV173923 standard to the sample.

Smith *et al.* [7] gave evidence that beta-lactam ring hydrolysis during the degradation of Imipenem in aqueous solutions was followed by intermolecular reactions between beta-lactam and carboxylic groups giving dimeric derivatives. Therefore, several reference standards of dimeric compounds related to GV104326 were analysed under the same conditions. One of the standards, GV196359 (Fig. 6) yielded the same retention time (24 min) as the last radioactive peak in rat urine chromatograms.

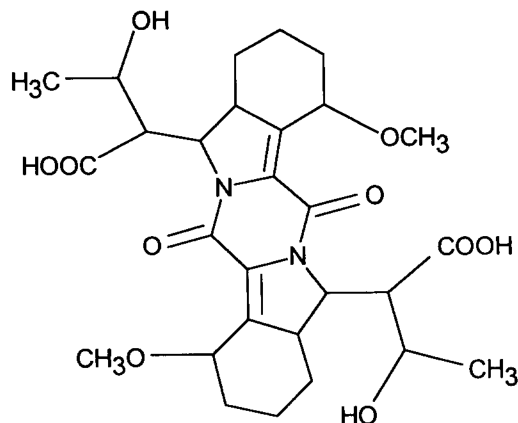

Figure 6
Structure of GV196359.

The urine samples were also analysed by HPLC–PB–MS. The component eluting at the same retention time as the parent compound showed a similar mass spectrum (Fig. 7) to that of GV104326 reference compound. The molecular ion occurred at *m/z* 281 and the other signals (e.g. *m/z* 253, 221) were due to fragment ions.

Furthermore, the mass spectra for the two major metabolites (Figs. 8 and 9) were in agreement with those of the standard compounds: for GV173923 the molecular ion occurred at *m/z* 267 and the decarboxylation product generated a fragment ion at *m/z* 223; GV196359 yielded a molecular ion at *m/z* 562 and several fragment ions were observed.

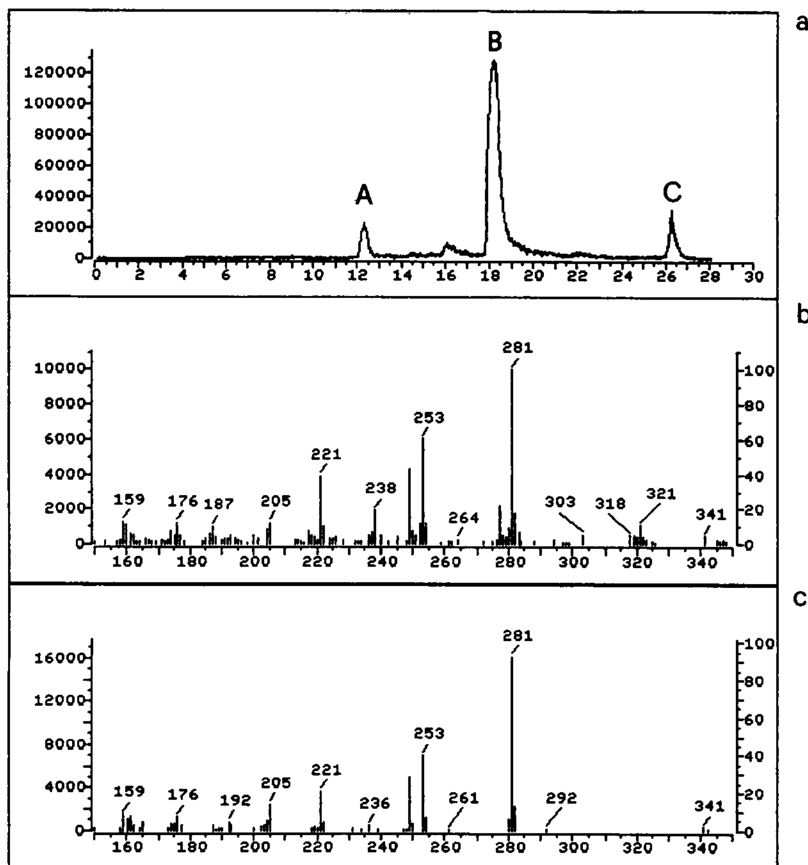

Figure 7
Particle beam/chemical ionization (NH_3, negative ions) data for: (a) total ion current (130–700 *m/z*) chromatogram of rat urine sample; (b) mass spectrum of peak "B" at 18.6 min; (c) mass spectrum of GV104326 reference standard.

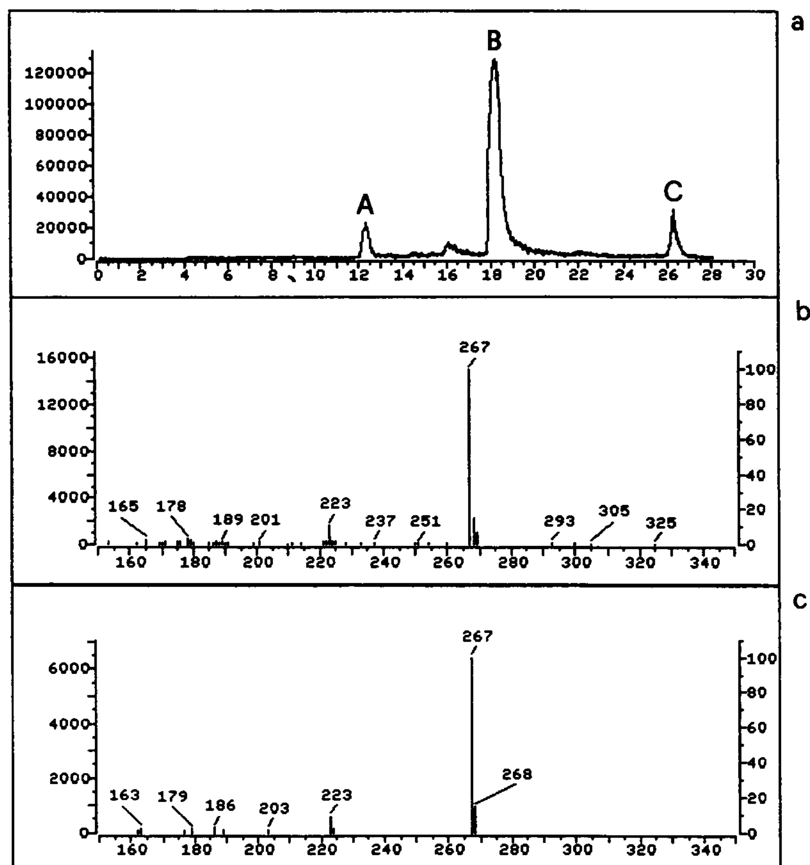

Figure 8
Particle beam/chemical ionization (NH_3, negative ions) data for: (a) total ion current (130–700 *m/z*) chromatogram of rat urine sample; (b) mass spectrum of peak "A" at 12.6 min; (c) mass spectrum of GV173923 reference standard.

Conclusions

Good recovery of GV104326 and its metabolites from rat urine samples was obtained by solid-phase extraction using SAX cartridges. Extracts were analysed by HPLC–particle beam–MS and HPLC with radioactivity detection. Rat urine samples were collected after a single intravenous dose of ^{14}C-GV104326 (50 mg kg^{-1}). The principal UV and radioactive peaks were confirmed by mass spectrometry to be related to the parent compound.

Two other peaks were identified by HPLC–PB–MS as an open beta-lactam ring derivative, GV173923, and a dimeric compound, GV196359. As these compounds were also observed after incubation of the parent drug in rat urine at 37°C (F. Pugnaghi, unpublished data), they were considered as potential metabolites and/or degradation products of GV104326 formed during the residence of urine in the bladder. Further work is planned to assess how these compounds are generated *in vivo*.

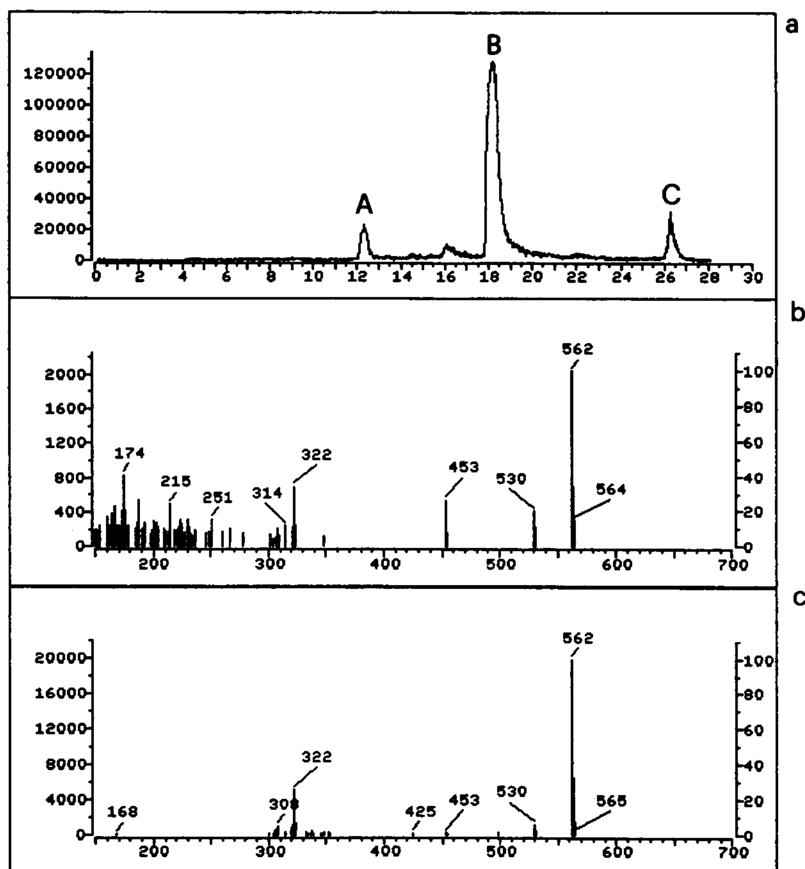

Figure 9
Particle beam/chemical ionization (NH_3, negative ions) data for: (a) total ion current (130–700 *m/z*) chromatogram of rat urine sample; (b) mass spectrum of peak "C" at 26.1 min; (c) mass spectrum of GV196359 reference standard.

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[*Received for review 21 September 1994; revised manuscript received 7 November 1994*]