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Short communication

Quantification of the bombesin/gastrin releasing peptide antagonist RC-3095 by liquid chromatography–tandem mass spectrometry

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Abstract

Bombesin (BN) and its mammalian equivalent, gastrin-releasing peptide (GRP), stimulate cell proliferation and are involved in the pathogenesis of several types of human cancer. BN/GRP and their receptors were shown to be critical for the growth of various human malignancies, such as small-cell lung, prostate, ovary, stomach and breast cancers in the human tumor xenograft model. In the present study, a fast, sensitive, robust method was developed for the determination and quantification of a BN/GRP receptor antagonist RC-3095 (D-Tpi-Gln-Trp-Ala-Val-Gly-His-Leuψ(CH2NH)Leu-NH2), in human plasma by liquid chromatography coupled with tandem mass spectrometry. RC-3095 was extracted from 0.2 ml human plasma by protein precipitation using cold acetonitrile (0.4 ml). The method has a chromatographic run of 10 min using a C_8 analytical column (150 mm × 4.6 mm i.d.) and the linear calibration curve over the range was linear from 20 to 10000 ng ml^{-1} ($r^2 > 0.994$). The between-run precision, based on the relative standard deviation replicate quality controls, was 5.7% (60 ng ml^{-1}), 7.1% (600 ng ml^{-1}) and 6.8% (8000 ng ml^{-1}). The between-run accuracy was ±0.0, 2.1 and 3.1% for the above-mentioned concentrations, respectively. The developed procedure allows the quantitative determination of peptide RC-3095 for pharmacokinetics studies in human plasma.

Keywords: RC-3095; Bombesin antagonist; Peptide; Human plasma; Quantification

1. Introduction

Bombesin (BN) and its mammalian counterpart, gastrin releasing peptide (GRP) have a wide range of neuroendocrine activities [1–2], such as proliferation of several cell types. Chronic administration of BN/GRP increases the DNA content of gastric and colonic mucosa and induces hyperplasia of mucosal cells [2]. Different studies have shown that antagonists of BN/GRP inhibit the growth of various cancers by interfering with the growth-stimulatory effects of bombesin-like peptides, and reducing the levels of epidermal growth factor [3–4]. RC-3095 belongs to series of BN/GRP receptor antagonists synthesized by Schally and co-workers [5], which was selected for clinical development due to its marked antitumor activity in experimental human malignancies in vivo and lack of significant side-effects in animal toxicology studies [6]. Little is known about the pharmacokinetics of BN antagonists. Several reports show that the half-time of BN-like peptides in circulation is very short. In rats the levels of a bombesin/GRP receptor antagonist [(D-Tpi-Gln-Trp-Ala-Val-Gly-His-Leuψ(CH2NH)Leu-NH2); Tpi = 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid], RC-3095, declined rapidly after a single subcutaneus administration and became undetectable after 3–5 h [2]. The radioimmunoassay is often used for quantification of BN receptor antagonist RC-3095 in plasma. However, this approach is laborious and needs selective antibodies

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against this peptide. Quantification of different drugs in biological matrices by liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) is becoming in common, due to the improved sensitivity and specificity of this technique [7]. Presently, RC-3095 is undergoing phase I clinical evaluation as a daily subcutaneous administration in patients with advanced refractory solid tumors. Considering that a previously described radioimmunoassay method for the measurement of bombesin/GRP antagonists proved to be inadequate for the measurements of RC-3095 in patient plasma samples [8], we have succeeded in developing a specific, sensitive and fast LC–MS–MS method for its quantification in human plasma. The procedure requires a simple protein precipitation and was developed for pharmacokinetics studies.

2. Experimental

2.1. Chemicals and reagents

BN receptor antagonist RC-3095 (99% of purity) was obtained from Zentaris (Frankfurt/Main, Germany). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA) while the formic acid, analytical grade was purchased from Merck (Rio de Janeiro, Brazil). Ultrapure water was obtained from a Gradient Millipore system (São Paulo, Brazil). Blank blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −70 °C until needed.

2.2. Calibration standards and quality controls

The stock solution of BN receptor antagonist RC-3095 was prepared in water at concentrations of 1 mg ml^{-1}. Calibration curves for BN receptor antagonist RC-3095 were prepared in blank human plasma at concentrations of 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10,000 ng ml^{-1} and performed in duplicate in each batch. Quality control samples were prepared in blank plasma at concentrations of 60, 600 and 8000 ng ml^{-1} (QCA, QCB and QCC, respectively).

2.3. Sample preparation

Aliquots (0.20 ml) of human plasma were employed for a simple protein precipitation, 0.40 ml of cold acetonitrile were added and the samples were vortex mixed for 40 s, centrifuged at 3000 g during 1 min, the upper layer were transferred into glass microvials, capped and placed in an autosampler.

2.4. Liquid chromatography and mass spectrometry conditions

An HPLC system (Hewlett-Packard, Model 1100) consisting of a binary pump (G1312A) was used for all analysis.

Table 1
Conditions of mobile phase gradient

Time (min)	Mobile phase A (%) (water, 0.1% TFA)	Mobile phase B(%) (acetronitrile, 0.1% TFA)
0.0	90	10
0.1	90	10
3.0	50	50
8.5	30	70
9.5	90	10
10.0	90	10

The chromatographic system consisted of a C_8 Genesis analytical column (100 mm × 4.6 mm id, 3 μm film thickness) and the mobile phase was composed of a mixture of solvent A [water (0.1% of TFA)] and B [acetonitrile (0.1% of TFA)] in a gradient as shown in Table 1. The total run time was set for 10 min at a flow rate of 1.0 ml min^{-1}. The column was operated at room temperature and present a void time of 1.0 min. The temperature of the autosampler (CTC Analytics, HTS PAL) was maintained at 6.5 °C and was set up to make 40 μL sample injection. Mass spectrometry was performed in a Sciex API 4000 triple stage quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA) equipped with an API electrospray source operating in pos-

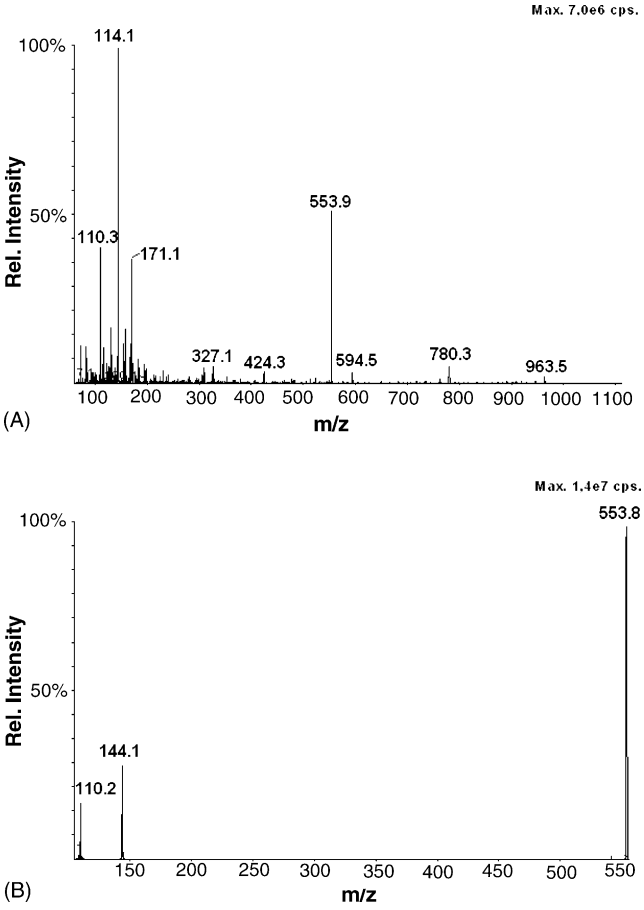

Fig. 1. Full-scan mass spectra (Q1) of the peptide RC-3095 (A) and their respective product ion spectra (B).

itive mode (ES+). The source block temperature was set at 450 °C and the electrospray capillary voltage to 5.5 kV, and nitrogen was used as a collision gas. The ions monitored in multiple reaction monitoring (MRM) in the conditions were declustering potential (DP) = 31 V, collision energy (CE) = 45 eV and collision exit potential (CXP) = 10 V; MRM *m*/*z* 553.9 → 144.1, was used for quantitation of BN receptor antagonist RC-3095. Data were acquired by Analyst software (1.3.1, Applied Biosystems) and calibrations curves for the analyte were constructed using the RC-3095 peak-area via a weighted ($1/x^2$) least-squares linear regression. Unknown sample peak-areas were then interpolated from the calibration curve to provide concentrations of BN receptor antagonist RC-3095.

2.5. Recovery

Preliminary experiments were conducted to evaluate the recovery with the extraction method described above. The percentage recovery was calculated as the ratio of the peak area for extracted blank plasma spiked at each standard concentration (60, 600 and 8000 ng ml^{-1}) relative to peak area of the equivalent blank plasma samples spiked after the extraction.

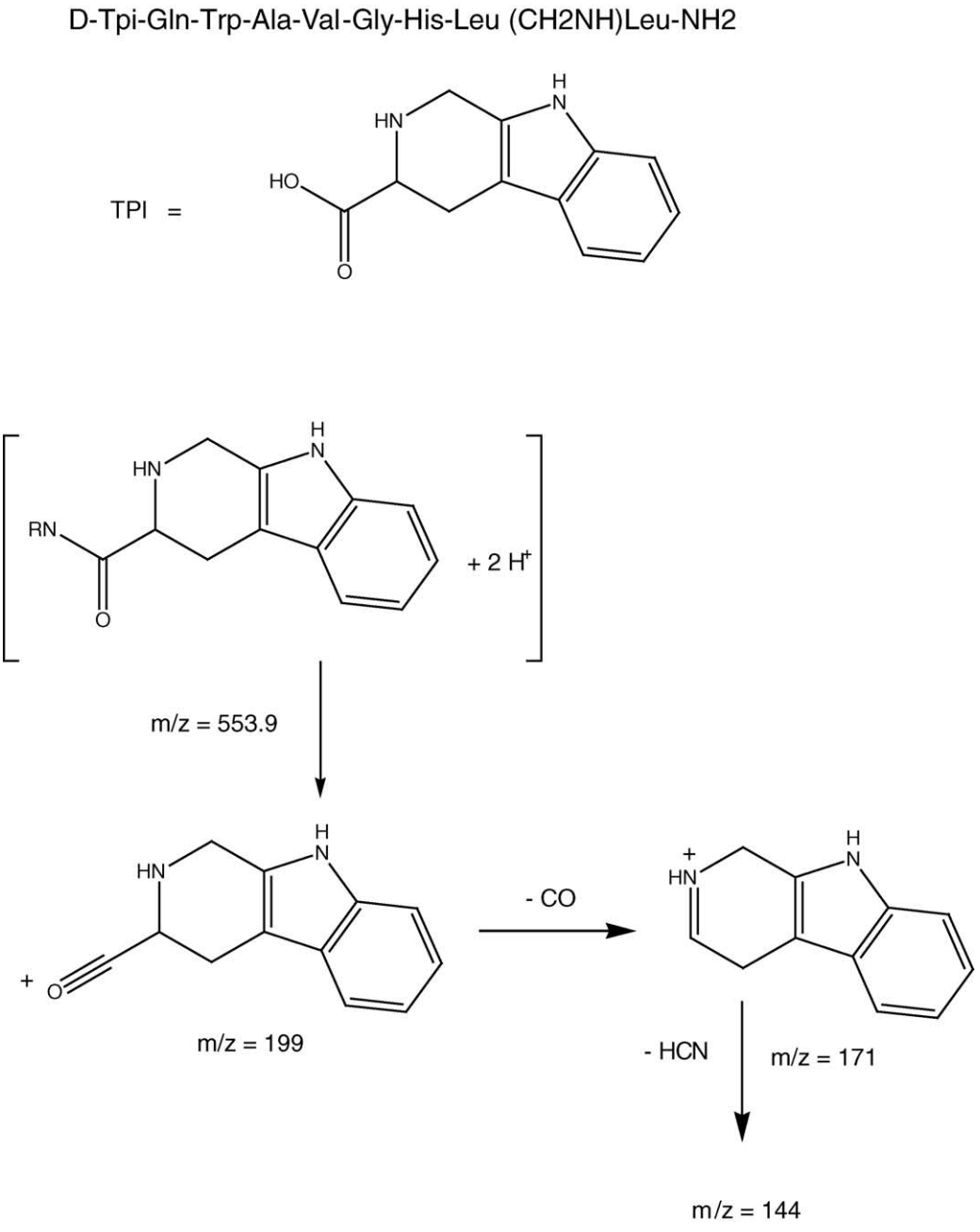

Fig. 2. Proposed mass fragmentation pathways for the peptide RC-3095.

2.6. Assessment of ion suppression

To assess the effect of ion suppression on the MS–MS signal of the analyte, RC-3095, the extraction procedure described above was evaluated. The experimental set-up consisted of an infusion pump connected to the system by a "zero volume tee" before the split and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of RC-3095. The infusion pump was set to transfer (10 μl/min) of the analyte in water (10 μg/ml).

2.7. Stability

Quality control samples prepared to test stability (60, 600 and 8000 ng ml^{-1}) were subjected to short-term (6 h) room temperature, three freeze–thaw cycles and 24 h autosampler (8 °C) stability tests. Subsequently the peptide RC-3095 concentrations were measured in comparison with freshly prepared samples.

2.8. Precision and accuracy

The within- and between-run precision were determined as the relative standard deviation, R.S.D.(%) = 100(S.D./M) and the accuracy as the percentage relative error, RE(%) = $(E - T)$ $(100/T)$, where M is the mean, S.D. is the standard deviation of M, E is the experimentally determined concentration and T is the theoretical concentration.

3. Results and discussion

3.1. Method development

Electrospray positive mass spectrum for BN receptor antagonist RC-3095 showed the ion m/z 144 as the base peak, other intense ion is the m/z 553.9 [(with relative intensity of 55%), molecular ion with two H^+] (Fig. 1A). The transition m/z 553.9–144.1 was the most intense transition observed in the MS–MS spectrum (Fig. 1B). The ion m/z 144.1 is produced by fragmentation of the Tpi group (2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid) (Fig. 2). Due to high intensity of the m/z 553.9 → 144.1 reaction and not detectable interference in blank human plasma samples this transition was used in the present method.

The limit of quantification (LOQ) was validated for 20 ng ml^{-1} and with a run time of less than 10.0 min. The mass chromatograms of a blank and LOQ samples are shown in Fig. 3, in which the retention time of RC-3095 was 4.9 min. No internal standard was used, since the preparation of the sample needs only a protein precipitation step.

In this system no was observed a ion suppression of the MS signal at the time where the analyte is eluted (Fig. 3C).

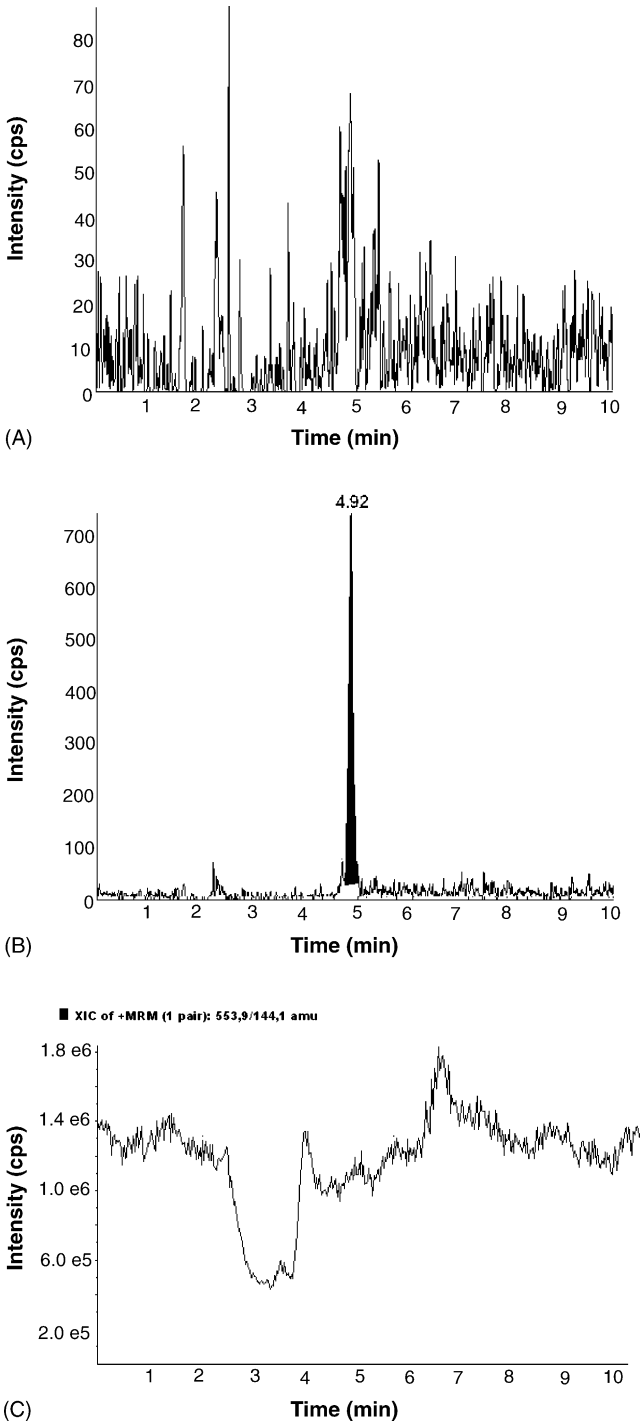

Fig. 3. MRM chromatogram for the peptide RC-3095 (A) of blank pooled human plasma, (B) of the peptide RC-3095 spiked in human plasma at a final concentration of 20 ng ml^{-1} and (C) the suppression ionic test for RC-3095 (infusion 10 μl ml^{-1} of a RC-3095 solution at 1 μg ml^{-1} in water).

3.2. Assay performance

The optimized method was validated by assessment of recovery, linearity, quantitation limit, precision and accuracy. Coefficients of variation and relative errors of less than 15% were considered acceptable, except for the quantitation

Table 2
Calibration curve of validation data for RC-3095

Expected value (ng/mL)	Validation curve results			Mean	Accuracy (%)	CV (%)
	Back concentration (ng/mL)	Back concentration (ng/mL)	Back concentration (ng/mL)			
20	18.9	19.1	20.2	19.4	97.0	3.6
	18.9	18.2	17.5	18.2	91.0	3.8
50	52.4	54	57.6	54.7	109.3	4.9
	56.9	57.6	50.9	55.1	110.3	6.7
100	109	107	106	107	107.3	1.4
	109	112	117	113	112.7	3.6
200	104	198	190	164	82.0	31.8
	105	206	207	173	86.3	33.9
500	484	514	504	501	100.1	3.1
	491	530	537	519	103.9	4.8
1000	1050	988	1060	1030	103.3	3.8
	1060	1010	1040	1040	103.7	2.4
2000	959	1960	1750	1560	77.8	33.9
	1010	2010	1910	1640	82.2	33.5
5000	4620	4700	4850	4720	94.5	2.5
	4770	4730	4830	4780	95.5	1.1
10000	9470	8630	8840	8980	89.8	4.9
	8520	8730	9040	8760	87.6	3.0
Correlation coeficient	0.9951	0.9990	0.9989			

limit (LOQ), whose values were extended to 20%, as recommended by Shah et al. [9] and Bressole et al. [10] for the analysis of biological samples for pharmacokinetics studies. The method was linear for the peptide from 20 to 10.000 ng ml^{-1} ($r^2 > 0.9970$) on repeated calibration curves, specific data as a mean of three calibrations curves are presented on the Table 2.

The recovery of RC-3095, calculated from the peak area ratios of extracted human plasma previously spiked at final concentrations of 60, 600 and 8000 ng ml^{-1} ($n = 15$ for each concentration) were 72.8, 89.7 and 87.4%, respectively.

Between- and within-run accuracy and precision for the quality controls are summarized in Table 3. In order to detect possible interferences in different plasma samples, the blank plasma samples of the 100 healthy volunteers were analyzed and in all cases, no peaks higher than 1/6 of the LOQ peak were observed. The result of these analysis was used to calculate the decision limit [11] of the present method (CCα, mean of the response obtained in 100 blank human plasma samples plus 2.33 S.D.) that was 3.6 ng ml^{-1}. The method offers advantages over those previously reported, in terms of a simple sample extraction and a faster run time (10 min).

BN/GRP and their receptors were showed to be critical for the growth of various human malignancies, such as small-cell lung, prostate, ovary, stomach and breast cancers in the human tumor xenograft model [12,13]. This autocrine stimulatory pathway has a direct effect on tumor cell proliferation and can be inhibited by a specific BN/GRP receptor antagonists [14]. Notably, the BN/GRP synthetic antagonist RC-3095 was devoid of any significant toxicity in tumor-bearing mice, at the doses in which tumor regressions were observed. For that reason, RC-3095 was selected as the first of a series of synthetic BN-GRP receptor antagonists to enter

Table 3
Validations with the quality controls (QC) having the results of the accuracy and precision of the peptide RC-3095

Parameter	Nominal concentration (ng ml^{-1})			
	20	60	600	8000
Intra-batch				
Mean found ($n = 8$) (ng ml^{-1})	21.6	66.3	537	7585
Accuracy (%)	7.7	2.5	1.6	2.2
Precision (%)	108.0	110.5	89.5	94.8
Inter-batch				
Mean found ($n = 3$) (ng ml^{-1})	21.1	67.5	585	7603
Accuracy (%)	3.8	1.4	5.8	3.5
Precision (%)	105.5	112.5	97.5	95.0

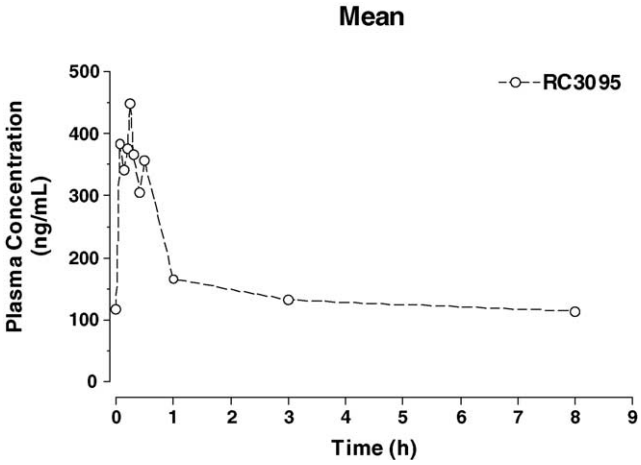

Fig. 4. Mean plasma levels of RC-3085 following subcutaneous administration two cancer patients (8–96 μg/kg).

human trials. Up to now, thirty patients with various types of advanced refractory malignancies received RC-3095 as part of a phase I clinical and pharmacokinetic study in which the drug was given to patients as a daily subcutaneous injection at doses ranging from 8 to 96 μg/kg. Evidence of tumor activity was documented in patients with advanced hormone-refractory prostatic and medullary carcinoma of the thyroid. Plasma was obtained from patients included in the study immediately prior and after RC-3095 administration at various time-points; preliminary results of two patients are showed in Fig. 4.

4. Conclusions

A LC–MS–MS method for the quantification of the peptide RC-3095 in human plasma was developed and validated. This method offers advantages over those previously reported, in terms of a simple sample extraction (protein precipitation without clean-up procedures) and a faster run time (10 min). The LOQ of 20 ng ml^{-1} is sufficient for the pharmacokinetics studies (the estimate LOQ/C_{max} < 3%) and could be further improved by sample concentration if required. The assay performance results indicate that the method is precise and accurate enough for the routine determination of the peptide RC-3095 in human plasma.

References

[1] F. Thomas, C. Mormont, B. Morgan, Drugs Future 19 (1994) 349.
[2] K. Szepeshazi, A.V. Schally, G. Halmos, N. Lamharzi, K. Groot, J.E. Horvath, Proc. Nat. Acad. Sci. U.S.A. 94 (1997) 10913.
[3] A.V. Schally, A.M. Comaru-Schally, A. Nagry, M. Kovacs, K. Szepeshazi, A. Plonowski, J.L. Varga, G. Halmos, G. Front, Neuroendocrinol 22 (2001) 248.
[4] A.M. Bajo, A.V. Schally, K. Groot, K. Szepeshazi, Br. J. Cancer 90 (2004) 245.
[5] Y. Qin, T. Ertl, R.Z. Cai, G. Halmos, A.V. Schally, Cancer Res. 54 (1994) 1035.
[6] J. Pinski, A.V. Schally, G. Halmos, K. Szepeshazi, K. Groot, Cancer Res. 54 (1994) 5895.
[7] R. Kostianien, T. Kotiaho, T. Kuuranne, S. Auriola, J. Mass Spectrom. 38 (2003) 357.
[8] K. Groot, J.E. Horvath, R.Z. Cai, A.V. Schally, Int. J. Pept. Protein Res. 45 (1995) 561.
[9] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilvery, J.P. Skelly, A. Yacobi, T. Layoff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pitman, S. Spector. Pharm. Res. 9 (1992) 588.
[10] F. Bressolle, M.B. Petit, M. Audran, J. Chromatogr. B 686 (1996) 3.
[11] COMMISSION DECISION (EC) 2002/657, Off. J. Europ. Communities L221 (2002) 8.
[12] A.M. Bajo, A.V. Schally, M. Krupa, F. Herbert, K. Groot, K. Szepeshazi, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 3836.
[13] H. Reile, P.E. Armatis, A.V. Schally, Prostate 25 (1994) 29.
[14] G. Halmos, J. Pinski, B. Szoke, A.V. Schally, Cancer Lett. 85 (1994) 111.