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Three complementary liquid chromatographic methods for determination of the peptoid cholecystokinin-B antagonist, CI-988, in rat plasma

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Abstract

Three different liquid chromatographic methods (two quantitative methods which employ fluorescence detection and one qualitative method which employs selected ion-monitoring detection) were developed and validated to provide complementary specificity for determination of CI-988, a cholecystokinin-B antagonist, in rat plasma. The first quantitative method involves isocratic separation of "non-ionized" CI-988 and internal standard on a C-18 column, whereas the alternative quantitative method involves isocratic separation of the "anionic" analytes. These two quantitative HPLC methods rely on the intrinsic fluorescence of CI-988 and internal standard for detection, and both methods are equally sensitive (linear range of 2.0–1000 ng ml^{-1}), accurate ($\pm 15\%$ relative error), and precise ($\leqslant 15\%$ relative standard deviation). Plasma CI-988 concentrations for samples ($N = 69$) assayed with the "non-ionized" separation are linearly correlated with concentrations for the same samples assayed with the "anionic" separation ($y = 1.08x - 0.57$, $R = 0.999$). In addition, a third qualitative method, HPLC–thermospray mass spectrometry, was developed to provide complementary evaluation of assay specificity through the use of selected CI-988 fragment ion monitoring. When investigating an anomalous chromatographic result that calls into question the specificity of a method, the availability and use of alternative validated chromatographic separations and orthogonal detection schemes are beneficial.

Keywords: CI-988; Cholecystokinin-B antagonist; Assay validation; Specificity; Correlation; Liquid chromatography; Bioanalytical method

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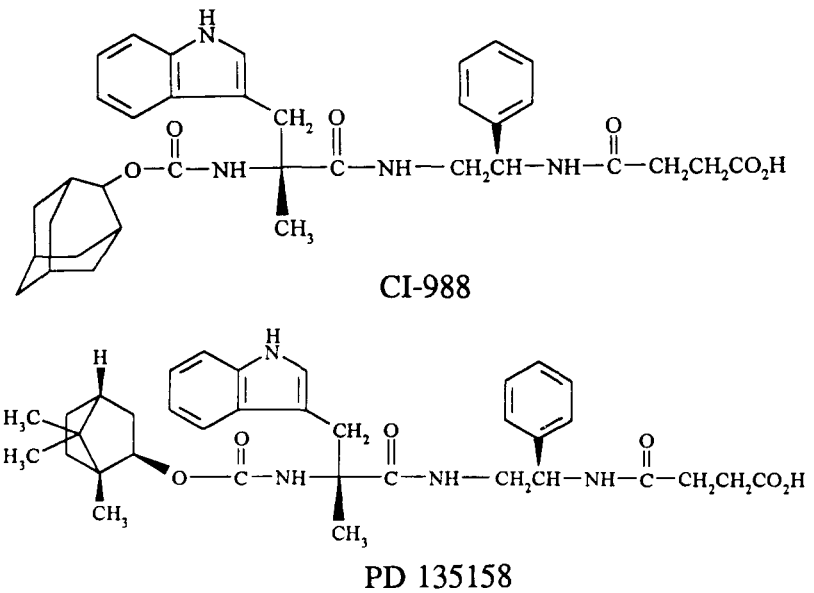

Fig. 1. Structures of CI-988 and I.S.

1. Introduction

Occasionally, during routine application of validated bioanalytical methods, one encounters samples with questionable chromatographic peaks. For example, appearance in control samples or predose samples of peak(s) with retention time(s) identical to analytes or possibly an anomalously high peak response for an analyte based on the analyte's pharmacokinetic profile. Further intrigue is added when these anomalous results are found to be repeatable and are not a result of analyst error or sample carryover. More often than not in such a situation, the reliability and specificity of the bioanalytical method is challenged.

Although evaluation of chromatographic specificity is a key component in any good analytical method validation protocol, whether it is a bioanalytical method (i.e. for bioavailability, bioequivalence and pharmacokinetic studies) [1–4] or not, assessment of assay specificity is an ongoing process. New sources of representative matrix are frequently encountered during routine application of the validated method. For each new pharmacokinetic, toxicokinetic, or even pharmacodynamic study, new subjects or test animals may be used. Matrix from each new subject or test animal represents a new independent source, and assay of these sample(s) provides a further test of the method's specificity and reliability. When confronted with specificity questions of an existing validated method, use of an alternative complementary method can often unequivocally distinguish between a true interference and a known analyte [5–7]. This paper describes three complementary liquid chromatographic methods for the determination of CI-988 in rat plasma.

CI-988 (Fig. 1) is a peptoid with nanomolar affinity for the cholecystokinin-B (CCK-B) receptor and micromolar affinity for the CCK-A receptor [8]. The CCK-B receptor, distributed primarily in the brain, is believed to be involved in the neuroregulation of symptoms associated with panic and anxiety [9–11]. CI-988 has demonstrated potent anxiolytic-like effects in animals [12,13] and recently showed modest activity in a clinical trial of CCK-4 induced panic in healthy subjects [14]. We have described a sensitive HPLC assay with fluorescence detection for CI-988 in human and monkey plasma [15].

The three liquid chromatographic separations described herein (two quantitative and one quali-

tative) provide complementary methods for determination of CI-988 (Fig. 2). CI-988 is a weak acid. The pK_a value for the terminal carboxylic acid group of 6.2 was determined by potentiometric titration (unpublished results), whereas a value of 4.3 was determined by standard aqueous solubility techniques and kinetic studies of CI-988 hydrolysis as a function of pH [16]. We have no explanation for the discrepancy in pK_a determinations. Nevertheless, chromatographic conditions were developed and validated for both chemical forms of CI-988. The non-ionized form of CI-988 (log P (octanol:water partition coefficient) of 4.6 in 0.1 N HCl) was separated in a mobile phase with an acidic (pH 3.5) aqueous component. In contrast, anionic CI-988 (log P of 2 in pH 7.5 phosphate buffer) was separated in a mobile phase with a pH 7.5 phosphate-buffered aqueous component. Specificity and accuracy of these complementary and independently-validated separations for CI-988 in rat plasma were verified in a correlation study. Furthermore, a third qualitative HPLC method that uses mass spectrometry with selected-ion monitoring proved a valuable complementary detection scheme for corroborating assay specificity, as described herein for CI-988.

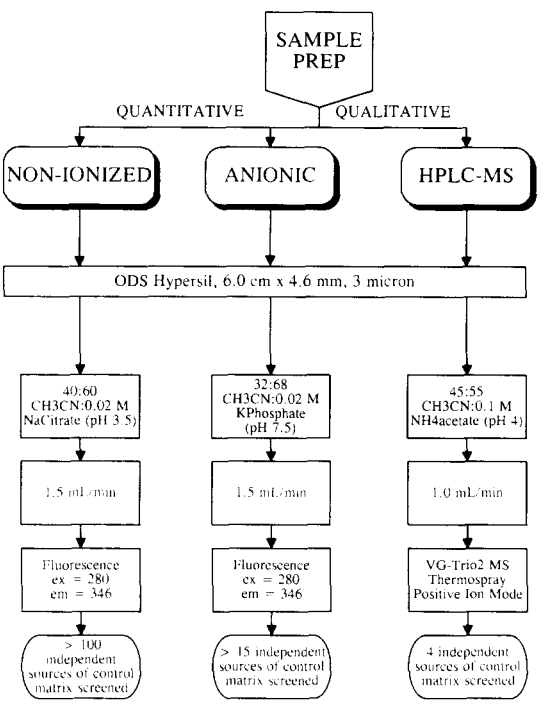

Fig. 2. Flow chart comparison of the three complementary liquid chromatographic methods for determination of CI-988 in rat plasma.

2. Experimental

2.1. Materials

The methylglucamine salts of CI-988 and the internal standard (PD 135158, I.S.) were synthesized by Parke-Davis Pharmaceutical Research. All concentrations are reported as CI-988 or I.S. free acid equivalents. CI-988 is known chemically as [*R*-(*R**,*R**)]-4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]-propyl]amino]-1-phenylethyl]amino]-4-oxo butanoic acid. PD 135158 is known chemically as 4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]-propyl]amino]-1-phenylethyl]amino]-4-oxo-[1*S*-[1α,2β,[*S**(*S**)],4α]]-butanoic acid.

Reagent grades of ammonium acetate, critric acid, 50% sodium hydroxide, hydrochloric acid, and isopropanol, and HPLC grades of acetonitrile, methanol, and water were obtained from EM Science (Cherry Hill, NJ). Reagent-grade potassium phosphates (mono- and dibasic) were obtained from Mallinckrodt (Paris, KT). Bond Elut® phenyl (1 ml × 100 mg) solid phase extraction cartridges were obtained from Varian Analytichem International (Harbor City, CA). Control heparinized rat plasma was obtained from Lampire Biologicals Laboratories (Pipers-ville, PA) and from Parke-Davis stock rats.

2.2. Standard preparation

Stock standard solution of 100 μg ml^{-1} CI-988 was prepared in methanol:water (4:6) and then serially diluted with water to give working calibration standard solutions of 2.0, 5.0, 10.0, 25.0, 50.0, 100, 250, 500 and 1000 ng ml^{-1}. Stock solution of I.S. (100 μg ml^{-1} PD 135158) was prepared in methanol and then diluted with water to give a working I.S. solution of 100 ng ml^{-1}.

2.3. Quality control sample preparation

Rat plasma CI-988 quality control (QC) samples were prepared at three different concentrations across the calibration standard range (low, mid, high) by diluting an appropriate aliquot of working stock CI-988 solution with control heparinized rat plasma such that the biological matrix content in the final sample was not less than 95%. QC samples were subdivided into 0.250 ml aliquots and stored frozen at −20°C in order to mimic conditions used for storing actual study samples.

2.4. Sample preparation

To 100 μl of rat plasma (sample, QC, or control) was added 100 μl of appropriate CI-988 working calibration standard solution or water and 100 μl of working I.S. solution or water. The sample was briefly mixed and then 0.5 ml of 0.1 N HCl was added. The resulting mixture was vortexed for about 10 s before being applied to the conditioned solid phase extraction cartridges.

2.5. Extraction procedure

The phenyl Bond Elut® cartridge was conditioned by washing with isopropanol (1.0 ml), methanol (1.0 ml), and water (1.0 ml). The sample was applied to, and drawn through, the cartridge with vacuum (≈5 in Hg). The sample tube was rinsed with 1.0 ml of water, which was then applied to, and drawn through, the cartridge. The cartridge was washed with 1.0 ml acetonitrile:water (10:90). After discarding the washings, the drug and I.S. were eluted in methanol (2 × 0.50 ml) into a clean tube. The eluate was evaporated to dryness under a stream of nitrogen in a water bath (≈40°C) (Turbo-Vap LV Evaporator, Zymark Corp., Hopkinton, MA). The residue was reconstituted in 250 μl mobile phase of which 180 μl was injected onto the HPLC column. For the thermospray mass spectrometry analysis, the residue was reconstituted in 100 μl mobile phase and all of it was injected.

2.6. Chromatographic conditions

The flow chart in Fig. 2 compares and contrasts the different chromatographic conditions used for the three complementary methods.

Fluorescence spectra were taken with a Perkin-Elmer LC-240 fluorescence detector. The HPLC–fluorescence system consisted of a Spectra Physics 8700 pump, Spectra Physics 8780 autosampler, a Perkin-Elmer LC-240 fluorescence detector (λ_{ex} = 282 nm, λ_{em} = 346 nm), and a Spectra Physics ChromJet integrator. The HPLC–mass spectrometer system consisted of a Waters 600MS pump connected in series to a VG-Trio 2 mass spectrometer. The mass spectrometer was operated in the thermospray positive ion mode, with a source temperature of 250°C, capillary temperature of 215°C, and discharge current of 0 μA. Full scans (700–150 Da) were taken repetitively at a scan rate of 1 s decade^{-1}. Selected-ion monitoring of m/z 615, 597, 515 and 363 was conducted with a dwell time of 0.8 s, interchannel delay of 0.1 s and 1 amu mass range.

All analytical separations were achieved isocratically on a 1.5 cm × 3.2 mm C-18 Newguard column from Applied Biosystems attached in series to an octadecylsilane (ODS) 3 μm Hypersil 6.0 cm × 4.6 mm column from Hewlett Packard (Palo Alto, CA). A C-18 scavenger cartridge column (3.3 cm × 4.6 mm, 8 μm) from Perkin Elmer (Norwalk, CT) was installed between the pump and autosampler during the anionic separation. The mobile phase for the non-ionized-based separation of CI-988 and I.S. with fluorescence detection consisted of a mixture of 0.02 M sodium citrate buffer (pH 3.5) and acetonitrile (60:40, v/v) at a flow rate of 1.5 ml min^{-1}. These chromatographic conditions are similar to those used in our previously reported assay for CI-988 in human and monkey plasma [15]. The mobile phase for the anionic separation with fluorescence detection consisted of 0.02 M potassium phosphate buffer (pH 7.5) and acetonitrile (68:32, v/v) at a flow rate of 1.5 ml min^{-1}. The mobile phase for the mass spectrometer method consisted of 0.1 M ammonium acetate (pH 4) and acetonitrile (55:45, v/v) at a flow rate of 1 ml min^{-1}. Under these conditions, the analytes would also be non-ionized.

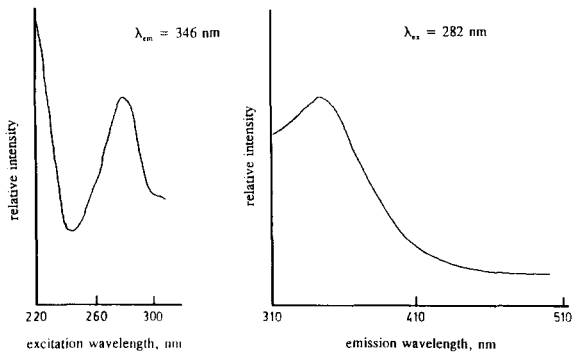

Fig. 3. Fluorescence emission and excitation spectra of CI-988.

2.7. Validation procedures

The two HPLC–fluorescence methods were validated over the concentration range 2.0–1000 ng ml $^{-1}$. For the non-ionized separation, nine calibration standards and three quality control samples were assayed in triplicate in three separate batch runs. For the anionic separation, the same nine calibration standards and three quality control samples were assayed in triplicate in one batch run. The best fit line was determined by linear regression analysis of calibration data with a weighting factor of 1/concentration squared. Samples were randomized prior to HPLC injection. Specificity, system parameters, recovery, accuracy, and precision were determined.

2.8. Stability

The stability of CI-988 was evaluated in stock solution (aqueous), mobile phase/injection solution, fresh rat plasma at 37°C, and frozen rat plasma stored at −20°C by comparing mean chromatographic responses of initial assays to those of subsequent assays. In addition, freeze–thaw stability of CI-988 QC samples was evaluated after subjecting samples to three freeze–thaw cycles. The stability of I.S. was also evaluated in aqueous solution and mobile phase.

3. Results and discussion

Fluorescence excitation and emission spectra for CI-988 are depicted in Fig. 3. Emission arises from the tryptophan moiety of CI-988 and affords nearly a 50 fold improvement in detection sensitivity relative to ultraviolet absorbance. The excitation and emission profiles for the non-ionized and anionic forms of CI-988 are indistinguishable; this is attributed to the protonation being so far removed from the tryptophan chromophore.

3.1. Stability

CI-988 and I.S. are stable in aqueous solution under normal laboratory conditions for at least four weeks. CI-988 and I.S. are stable after extraction from rat plasma for at least 2 days at room temperature in mobile phase. No loss of CI-988 was evident after 24 h in fresh rat plasma maintained at 37°C. Furthermore, CI-988 is stable in frozen rat plasma for at least a year and is stable after at least three freeze–thaw cycles.

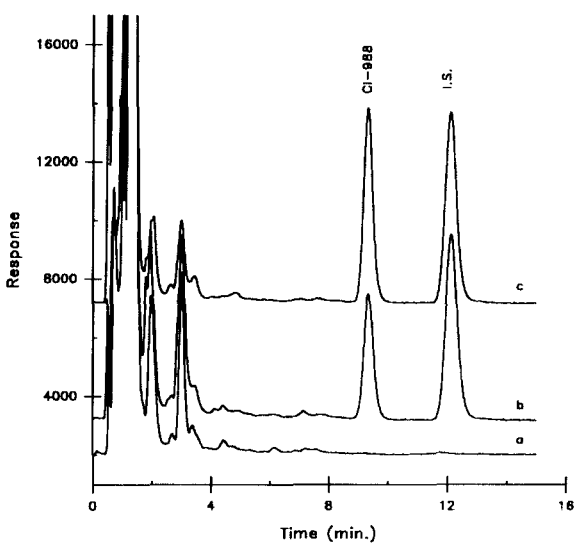

Fig. 4. Representative chromatograms for the non-ionized CI-988 separation (conditions described in text): (a) blank control rat plasma; (b) 50.0 ng ml $^{-1}$ CI-988 calibration standard in rat plasma; (c) pharmacokinetic study plasma sample (rat #54725).

Table 1
Comparison of system parameters [a] for non-ionized and anionic chromatographic separations of CI-988 with fluorescence detection

System parameters	Non-ionized	Anionic
CI-988		
RT (min)	9.5 ± 0.5	6.1 ± 0.2
N	4210	5590
k'	14	11.2
Internal standard		
RT (min)	12.2 ± 0.6	8.6 ± 0.3
N	5450	6860
k'	18.6	15.8
α	1.3	1.4

[a] *RT* = retention time; *N* = theoretical plates; *k'* = capacity factor; α = separation factor.

3.2. Non-ionized CI-988 separation

Representative chromatograms (blank plasma, standard and sample) from the non-ionized CI-988 separation are shown in Fig. 4. No rat plasma components elute at or near the retention time of CI-988 (9.5 min) or I.S. (12.2 min). Table 1 summarizes the system parameters. CI-988 and I.S. are suitably resolved from each other ($\alpha = 1.3$) and from other rat plasma components. In addition, more than 100 independent sources of control rat plasma have been assayed with this method during either validation or routine sample analysis, and no interfering peaks have been observed. This number of 100 sources is contrasted with the minimum of six independent sources of matrix to be screened during assay development and validation recommended by the 1990 conference on *Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies*, co-sponsored by AAPS, the FDA, Federation International Pharmaceutique, Health Protection Branch of Canada, and the Association of Official Analytical Chemists [1].

The method was validated by assaying rat plasma calibration standards over the range 2.0–1000 ng ml^{-1} CI-988 in triplicate in three separate batch runs. Peak-height ratios (CI-988/I.S.) were proportional to the amount of CI-988 added to rat plasma over this range ($y = 0.0138x - 0.001$; $R = 0.998$). Inter-batch calibration curve reproducibility, defined as the variation about the mean ($N = 9$) calculated standard concentration, ranged from 1.2% to 6.3% relative standard deviation (RSD). Assay precision and accuracy were determined by assaying three quality control samples in triplicate in each of three separate batch runs. Assay precision for CI-988 was ≤3.6%, based on % RSD values of 3.6, 3.0 and 2.7 at concentrations of 25.0, 250 and 800 ng ml^{-1}. Assay accuracy was within ±11.5%, based on relative errors (REs) of −0.8%, 8.4% and 11.5% for calculated concentration with respect to nominal concentration at the same respective concentration. The lower limit of quantitation (LLOQ), defined as the lowest concentration on the standard curve which could be quantified with acceptable precision (≤ 10% RSD) and accuracy (≤10% RE), was 2.0 ng ml^{-1}.

The absolute recovery of CI-988 in plasma was determined by comparing the peak height response of CI-988 in extracted plasma samples to mean peak height response of unextracted standards at comparable concentrations that were prepared in mobile phase. Mean (±SD) recovery was 90 (±5)% over the calibration concentration range.

3.3. Anionic CI-988 separation

Representative chromatograms (blank plasma, standard and sample) from the anionic CI-988 separation are shown in Fig. 5. No rat plasma components elute at or near the retention time of CI-988 (6.1 min) or I.S. (8.6 min). System parameters are compared to those for the non-ionized CI-988 separation in Table 1. It is worth noting that the retention times and capacity factors (*k'*) for both CI-988 and I.S. have decreased relative to those for the non-ionized separation even though the percentage of acetonitrile in the mobile phase has been reduced from 40% to 32%. This decrease in *k'* and corresponding increase in theoretical plates (*N*) is attributed to the increase in polarity and hydrophilicity of the anions relative to the non-ionized species [17]. Under these conditions, CI-988 and I.S. are well resolved from each other ($\alpha = 1.4$) and from other rat plasma

components. Specificity of this separation has been demonstrated by the absence of interfering peaks during assay of more than 15 independent sources of control rat plasma.

This method was validated by assaying rat plasma calibration standards over the same range of 2.0–1000 ng ml^{-1} CI-988 in triplicate in one batch run. One validation batch run was deemed sufficient because the only part of the method that was changed was the chromatographic separation. The extraction procedure, which introduces the greatest variability in the method, remained the same, and therefore the absolute recovery of CI-988 should not be affected. Validation parameters are compared to the non-ionic separation in Table 2. Peak-height ratios (CI-988/I.S.) were proportional to the amount of CI-988 added to rat plasma in this range ($y = 0.0135x - 0.002$; $R = 0.999$). Calculated concentrations of the three QC samples previously validated with the non-ionic-based separation were well within acceptance criteria for accuracy (±10% RE) with an intra-run precision of <3% RSD. Overall assay precision and accuracy were determined by compiling the QC results from the validation batch run with the QC results (assayed in duplicate) from three sample batch runs ($N = 9$). Assay precision for CI-988 was ≤12.7% RSD; assay accuracy was within ±6.8% RE. The LLOQ of 2.0 ng ml^{-1} was the same as for the non-ionic separation. Mean (±SD) absolute recovery for CI-988 of 86 (±4)% was also unchanged, as expected.

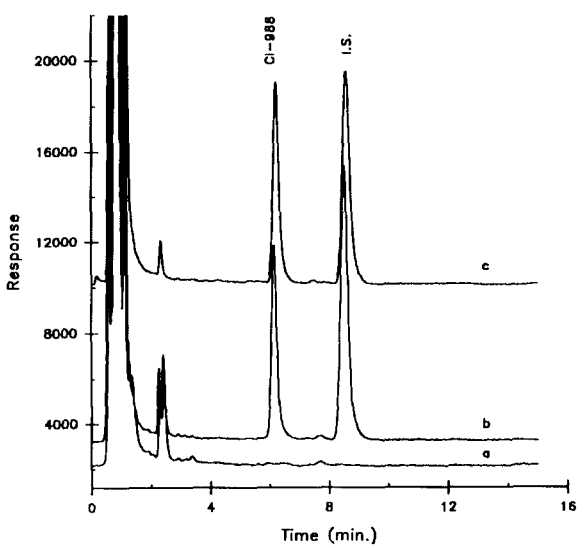

Fig. 5. Representative chromatograms for the anionic CI-988 separation (conditions described in text): (a) blank control rat plasma; (b) 50.0 ng ml^{-1} CI-988 calibration standard in rat plasma; (c) pharmacokinetic study plasma sample (rat #54725).

Table 2
Comparison of validation parameters[a] for non-ionized and anionic chromatographic separations of CI-988 with fluorescence detection

CI-988 Validation parameters	Non-ionized	Anionic
Plasma volume (μl)	100	100
Linear range (ng ml^{-1})	2.0–1000	2.0–1000
LLOQ (ng ml^{-1})	2.0	2.0
Assay accuracy (% RE)	±11.5	±6.8
Assay precision (% RSD)	≤3.6	≤12.7
Mean (±SD) absolute recovery	90(±5)%	86(±4)%

[a] LLOQ = lower limit of quantitation; % RE = percent relative error; % RSD = percent relative standard deviation.

3.4. Correlation

A correlation study was conducted to compare plasma CI-988 concentrations determined with the anionic CI-988 separation to plasma concentrations determined with the non-ionized CI-988 separation. 69 rat plasma samples from pharmacokinetic studies were assayed by both methods. Plasma concentrations are linearly correlated as can be seen in Fig. 6. Linear regression of the 69 pairs of concentration values gives an equation of $y = 1.08x - 0.57$ ($R = 0.999$). Comparable accuracy, precision and effective sensitivity (calibration range) of both separations are further demonstrated by the slope of 1.08 (standard error of ±0.003) being essentially equivalent to the theoretical value of unity [18]. The nearly-zero intercept (standard error of ±0.94) indicates negligible systematic bias in either method.

Both methods were used together to confirm or dispute the presence of CI-988 in some questionable rat plasma samples from toxicokinetic studies. Samples that produced HPLC peaks at

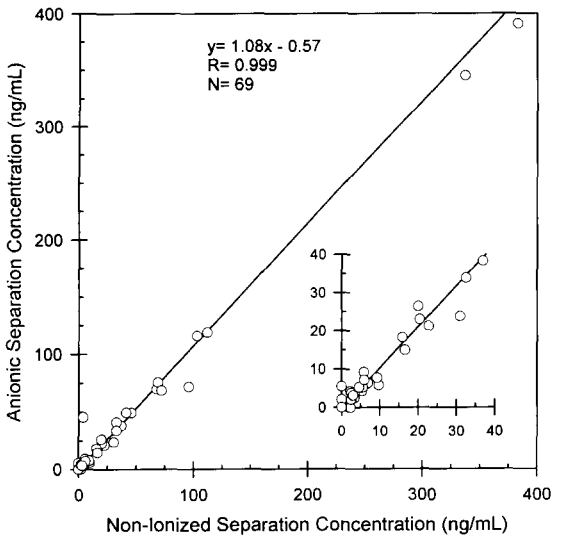

Fig. 6. Correlation plot of plasma CI-988 concentrations determined with the non-ionized separation vs plasma CI-988 concentrations determined with the anionic separation ($N = 69$). The insert is an expanded view of the lower concentration range.

identical retention times to those of CI-988 standards under both chromatographic separations and that produced peak responses (i.e. concentrations) which matched (i.e. linearly correlated) were reported to contain CI-988. Questionable samples that did not produce matching retention times nor responses were not encountered.

3.5. HPLC–mass spectrometry

Fig. 7 shows the thermospray positive ion mass spectrum of CI-988 obtained at a retention time of about 6.5 min (≈scan 105) following HPLC separation as described above. Present in the spectrum is the protonated molecular ion at m/z 615, loss of water at m/z 597, loss of the $COCH_2CH_2COOH$ sidechain at m/z 515, and loss of the adamantol ($C_{10}H_{14}O$) and $COCH_2CH_2$ COOH fragments at m/z 363. Due to the lack of sensitivity in the scan mode, three ions (m/z 615, 597 and 515) were used for selected-ion monitoring analysis.

Figs. 8 and 9 show the selected-ion monitoring chromatograms of an extracted 125 ng ml^{-1} CI-988 rat plasma standard (total mass of 12.5 ng in 100 μl) and extracted control rat plasma sample respectively. For the extracted CI-988 standard, responses are obtained for the three selected ions at the expected retention time for CI-988 with signal-to-noise ratios of >10:1. The control rat plasma sample was injected immediately after the CI-988 standard. No response was seen in any of the selected ions at the expected retention time for CI-988 (between 6 and 7 min), which shows the lack of endogenous components with interfering ions and the absence of any carryover from the previously injected CI-988 plasma standard. Additionally, three other independent sources of con-

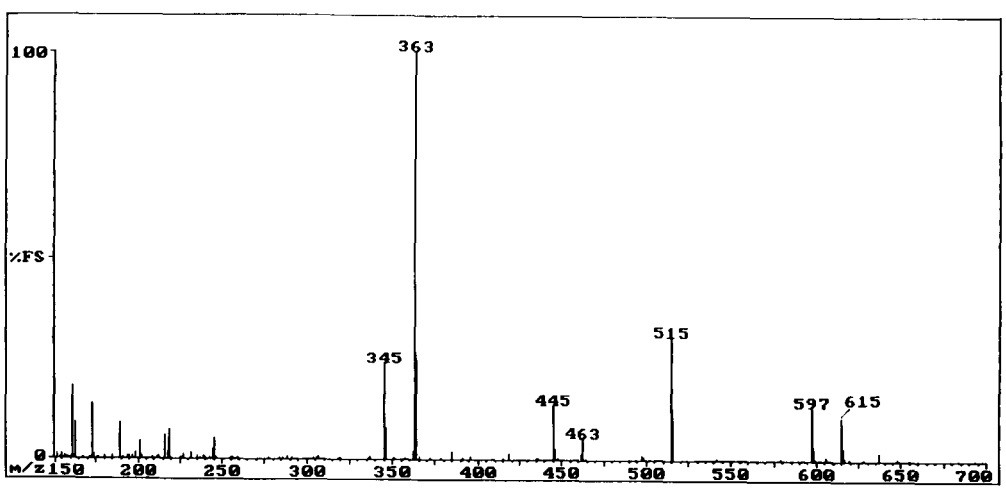

Fig. 7. Thermospray positive ion mass spectrum of CI-988 (conditions described in text).

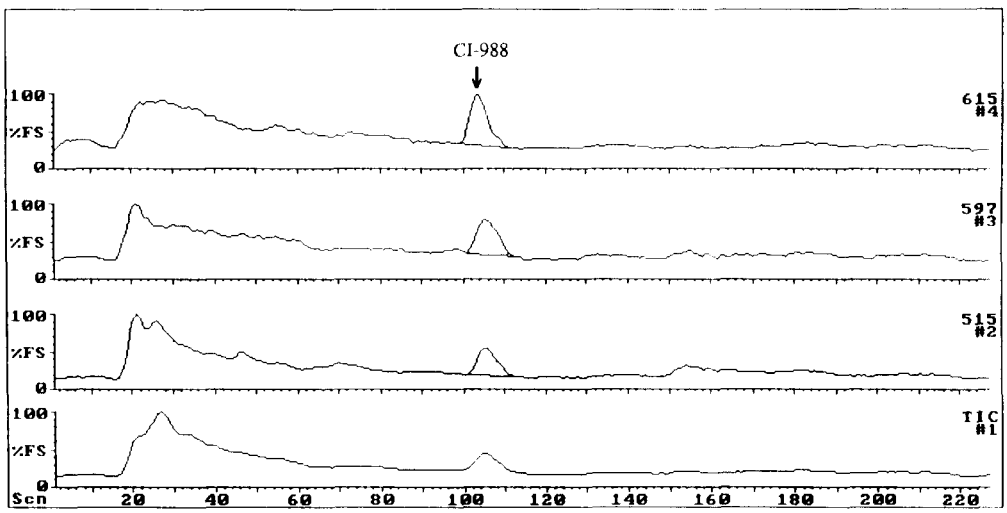

Fig. 8. Thermospray selected-ion monitoring chromatograms of a CI-988 calibration standard (12.5 ng) in rat plasma. Chromatograms from top to bottom: *m/z* 615, *m/z* 597, *m/z* 515, and total ion.

trol rat plasma were screened with this method and found to be free of interfering ions at the retention time for CI-988.

The method was used to qualitatively evaluate questionable rat plasma samples from toxicokinetic studies. The presence of CI-988 was confirmed or denied due to the presence or absence of ion peaks in the same relative proportion and the same retention time as standard reference materials.

Overall, six "anomalous" samples were assayed with all three methods. For all six samples, responses with retention times consistent with CI-988 were observed with all three methods. In addition, responses were linearly correlated between the two quantitative methods, and relative proportions of ion peaks in the HPLC–MS method were similar to CI-988 standards. On the basis of these findings, the presence of CI-988 was verified in these "anomalous" samples, and "interferences" were ruled out.

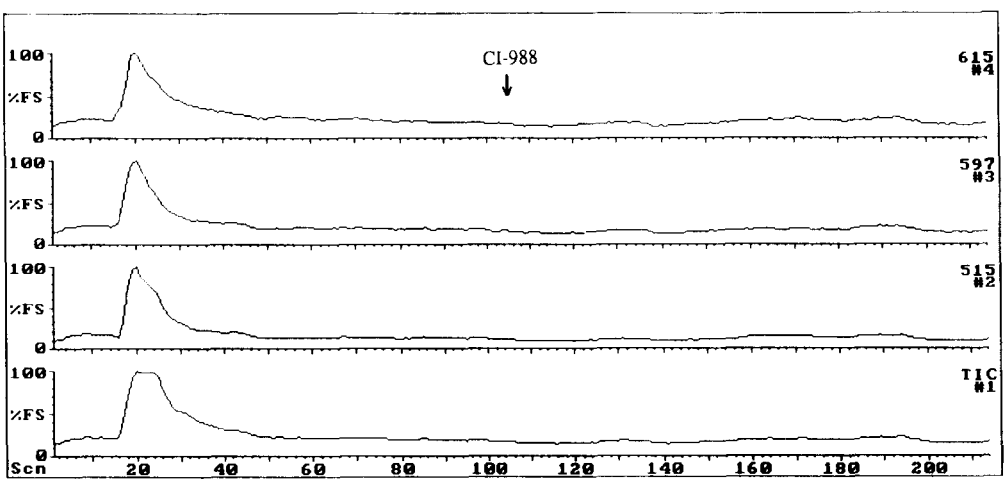

Fig. 9. Thermospray selected-ion monitoring chromatograms of control blank rat plasma. Chromatograms from top to bottom: *m/z* 615, *m/z* 597, *m/z* 515, and total ion.

4. Conclusions

When investigating an anomalous chromatographic result that calls into question the specificity of the method, the availability of alternative validated methods is essential. Use of complementary chromatographic separations and orthogonal detection schemes can often unequivocally distinguish between an analyte and an interferant. Complementary separations of CI-988 (non-ionized and anionic) were developed based on the weak acid dissociation constant of CI-988. In addition, selected-ion monitoring by thermospray mass spectrometry proved useful as an orthogonal detection mode to the instrinsic fluorescence of CI-988.

References

[1] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 81 (1992) 309–312.

[2] L.A. Pachla, D.S. Wright and D.L. Reynolds, J. Clin. Pharmacol., 26 (1986) 332–335.

[3] H.T. Karnes, G. Shiu and V.P. Shah, Pharm. Res., 8(4) (1991) 421–426.

[4] D. Dadgar, P.E. Burnett, M.G. Choc, K. Gallicano and J.W. Harper, J. Pharm. Biomed. Anal., 13 (1995) 89–97.

[5] J. Kirschbaum, S. Perlman, J. Joseph and J. Adamovics, J. Chromatogr. Sci., 22 (1984) 27–30.

[6] E. Gelpi, Life Sci., 41 (1987) 849–852.

[7] L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, 2nd edn., John Wiley, New York, 1979, pp. 591–614.

[8] J. Hughes, P. Boden, B. Costall, A. Domeney, E. Kelly, D.C. Horwell, J.C. Hunter, R.D. Pinnock and G.N. Woodruff, Proc. Natl. Acad. Sci. U.S.A., 87 (1990) 6728–6732.

[9] G.N. Woodruff, D.R. Hill, P. Boden, R. Pinnock, L. Singh and J. Hughes, Neuropeptides, 19 (1991) 45–56.

[10] L. Singh, A.S. Lewis, M.J. Field, J. Hughes and G.N. Woodruff, Proc. Nat. Acad. Sci. U.S.A., 88 (1991) 1130–1133.

[11] J. Hughes, J.C. Hunter and G.N. Woodruff, Neuropeptides, 19 (1991) 85–89.

[12] L. Singh, M.J. Field, J. Hughes, R. Menzies, R.J. Oles, C.A. Vass and G.N. Woodruff, Br. J. Pharmacol., 104 (1991) 239–245.

[13] K.R. Powell and J.E. Barrett, Neuropeptides, 19 (1991) 75–78.

[14] J. Bradwejn, D. Koszycki, M. Paradis, P. Reece, J. Hinton and A. Sedman, Biol. Psychiatry, 38 (1995) 742–746.

[15] J.P. Hinton, K. Jennings, E.L. Johnson and D.S. Wright, Biomed. Chromatogr., 9 (1995) 94–97.

[16] A.S. Kearney, S.C. Mehta and G.W. Radebaugh, Pharm. Res., 9 (1992) 1092–1095.

[17] L.R. Snyder, J.L. Glajch and J.J. Kirkland, Practical HPLC Method Development, John Wiley, New York, 1988, pp. 100–114, 126–130.

[18] H.A. Strobel and W.R. Heineman, Chemical Instrumentation: A Systematic Approach, 3rd edn., John Wiley, New York, 1989, p. 355.