Isolation and Characterization of a Felinine Containing Peptide
from the Blood of the Domestic Cat (Felis catus)

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Running title: γ-glutamylfelinylglycine: a peptide isolated from cat blood
SUMMARY

Felinine is a unique sulphur containing amino acid found in the urine of domestic cats and select members of the Felidae family. Research over the past 50 years has led to the conclusion that felinine must be synthesised in the kidney, as free felinine is not present in the blood or tissues of cats. We propose that felinine is present in the blood as $\gamma$-glutamylfelinylglycine, a glutathione conjugate. To test our hypothesis $^{35}$S-cysteine was administered intraperitoneally to one entire male cat, and two radiolabelled fractions were isolated from the blood. We showed that the amounts of both fractions in serum were linked to the gender of the cat, with entire males expressing significantly higher levels compared to castrated males, entire females or spayed females. Both fractions were characterized using amino acid analysis with one fraction (S18), containing an equimolar ratio of cys, glu and gly, while fraction S16, was found to contain cys, plus free amino acids. Nanospray mass spectrometry confirmed the sequence of fraction S18 as being $\gamma$-glutamylfelinylglycine, and conclusively proving that felinine is present in the blood of cats as part of a larger molecule, thereby questioning the current theory that felinine is synthesized in the kidney.
INTRODUCTION

Felinine is a sulphur containing amino acid that has been found in the urine of certain members of Felidae. In the domestic cat (*Felis catus*), the entire male has been found to produce the greatest amount of urinary felinine (122 µmol/kg/day compared to 41, 36 and 20 µmol/kg/day in castrated males, entire females and castrated females, respectively (1)). The biological role of felinine in the cat remains unknown but it has been postulated that it may be a precursor to a pheromone (2).

Since its discovery in 1951, many workers have attempted to detect felinine in various tissues and body fluids of the cat. Both Westall (3) and Datta and Harris (4) failed to detect the presence of free felinine in the plasma of cats. Tallan *et al.* (5) detected trace amounts of felinine in the plasma and in a bladder tissue extract but none in the kidney, liver or brain. Roberts (6) found trace amounts of felinine in the kidney, liver and skin. The general failure to detect felinine in tissues of the cat has led to the conclusion that the kidney is the likely site of felinine synthesis.

Using more sensitive methods for detecting felinine, Hendriks *et al.* (7) reported that felinine was detectable in the kidney, urine and bladder of the domestic cat, but that felinine could not be found in the liver, skin, blood, intestines, pancreas or the spleen.

There is convincing evidence that felinine is synthesized from the same isoprenoid pool as cholesterol (8). The formation of felinine *in vivo* can occur through a condensation reaction of an allylic carbonium ion and cysteine. In a previous study we have shown that radioactivity from $^{35}$S-cysteine and $^{35}$S-methionine is incorporated into felinine (7), while DL-2-$^{14}$C-leucine, DL-2-$^{14}$C-mevalonic acid, and 2-$^{14}$C-acetate have been shown to be incorporated into felinine by Avizonis and Wriston (9), Shapiro (10) and Wang (11).
A proposed metabolic pathway for the biosynthesis of felinine in the domestic cat, describes felinine being formed from isopentenylpyrophosphate (IPP) and cysteine, possibly in the liver (10, 11, 12, 13). We propose felinine arises from a conjugation reaction between the cysteine in glutathione (GSH) and IPP to form the tripeptide $\gamma$-glutamylfelinylglycine, rather than free felinine being formed from free cysteine and IPP. This mechanism would be similar to that described for the formation of isovalthine, another unusual sulphur amino acid that has been isolated from the urine of domestic cats, lions and humans suffering from hypothyroidism and hypercholesterolemia (14). Isovalthine has been shown to be formed in vitro as part of a glutathione-isovaleric acid conjugate (15). In the case of the $\gamma$-glutamylfelinylglycine conjugate, following formation in tissues such as the liver the peptide could then be transported via the blood to the kidney, where free felinine could be liberated by the actions of $\gamma$-glutamyltransferase and aminopeptidase M, and then be excreted in the urine. Our hypothesis would explain the absence of free felinine in the blood of domestic cats.

The aim of this study was to determine if a felinine containing peptide is present in the blood of the domestic cat, and if so, to determine the structure of the peptide.
EXPERIMENTAL PROCEDURES

The studies reported here were approved by and conformed to the requirements of the Massey University Animal Ethics Committee (16).

Animals and Diet - Domestic short-haired cats (*Felis catus*), 4-7 years old from the Feline Unit at Massey University (Palmerston North, New Zealand) were used as experimental animals. The body weights of the cats at the start of the experiment ranged from 3.52 to 4.11kg. Throughout the study, the cats were fed a moist canned cat food that passed a minimum feeding protocol for proving an adult maintenance claim for a cat food (17). The diet provided the following nutrients (g/100g dry matter): crude protein 52, crude fat 29, methionine 1.2, cysteine 1.8, taurine 0.2, and calculated metabolisable energy of 461kcal/100g dry matter. All cats had been vaccinated against feline rhinotracheitis, calicivirus and panleukopenia using a modified live vaccine (Felocell CVR, Norden Laboratories, München, Germany). Feline leukemia and feline immunodeficiency virus have not been detected in the colony since its establishment in 1976.

Preparation of Cat Serum for Reversed-phase High Performance Liquid Chromatography (HPLC) Analysis - Approximately 1-5ml of blood was collected into a plain tube (Vacutainer, Becton Dickinson). The blood was allowed to clot for 2hr, before being centrifuged at 4,000rpm for 10min for the collection of the serum. The serum was then ultrafiltered through a VIVASPIN Concentrator (5,000 MW cut off) (VIVASCIENCE Ltd, Lincoln, UK) by centrifugation at 7,500rpm for 30min. The ultrafiltrate was used for further analysis by HPLC and the retentate discarded.
Analysis and Separation of Peptides by HPLC - Peptides present in the ultrafiltered serum were separated by reversed-phase HPLC performed on a Phenomenex Luna 5\(\mu\) column (4.6 x 150mm), using a Waters HPLC system (Waters Corporation, Milford, MA). A linear 75min gradient was run, from 100\% solvent I (0.1\% TFA) to 100\% solvent II (0.1\% TFA and 90\% acetonitrile), which commenced 15min after sample injection. Peptides were detected by absorbance at 214nm and were manually collected, as required. Where applicable, peaks were integrated using Millennium\textsuperscript{\textregistered} Chromatography Manager software (Waters Corporation, Milford, MA).

Quantitation of $^{35}$S-radioactivity - Radioactivity present in the $^{35}$S-cysteine injection solution, serum samples and fractions collected from reversed-phase HPLC were determined as described by Hendriks et al. (7).

Amino Acid Analysis - The amino acid content of the isolated peptides was determined on duplicate samples using a Waters ion-exchange HPLC system (Waters Corporation, Milford, MA), utilizing post-column ninhydrin derivatisation and detection at 570 and 440nm, following hydrolysis in 6M glass-distilled HCl containing 0.1\% phenol for 24hr at 110 $\pm$ 2$^\circ$C in evacuated sealed tubes. Cysteine, methionine and tryptophan are partially or completely destroyed during the acid hydrolysis stage using this method.

Mass Spectral Analysis - The mass spectral analyses were performed at the Division of Immunology, Beckman Research Institute of the City of Hope (Duarte, CA, USA), using
electrospray ionisation in the positive ion mode on a ThermoFinnigan LCQ Deca iontrap mass spectrometer. Tandem mass spectrometry (MS/MS) was based on collision-induced dissociation (CID). The sample, dissolved in 1:1 acetonitrile:water and 2% acetic acid was introduced into the mass spectrometer via a nanospray needle assembly.

*Experiment 1* - One entire male cat (3.8kg) was administered 1.85mL of sterile isotonic (9g/L) saline containing 250µCi (9.25MBq) $^{35}$S-cysteine (Amersham Corporation, Arlington Heights, IL) by intraperitoneal injection. The cat was individually housed in a metabolism cage with free access to food and fresh water during the experimental period. At time 0, 1, 2, 4, and 8hrs a 1ml blood sample was taken by jugular vein puncture, under anaesthetic (isoflurane). Serum was separated from each blood sample, and ultrafiltered prior to separation by HPLC as described previously. All the major peaks present in the chromatogram of each ultrafiltered serum sample, and the baseline regions between peaks were manually collected into preweighed tubes, after which the tubes were reweighed to determine the quantity of fluid collected prior to measurement of radioactivity.

*Experiment 2* - Blood samples were taken from cats of different gender by jugular vein puncture. Serum was separated from each blood sample, and ultrafiltered prior to separation and quantitation of peaks S16 and S18, by HPLC as described previously.

*Experiment 3* - Following an appropriate “cool down” period, blood from the cat used in Experiment 1 was obtained. Fractions S16 and S18 were isolated from ultrafiltered serum as previously described and used for characterization studies.
RESULTS

Identification of $^{35}$S-cysteine serum peptides - The results from Experiment 1 showed the presence of three major $^{35}$S containing peaks, which between them contained 65-99% of the radioactivity in the loaded serum sample, depending on the time the blood samples were collected after injection of the $^{35}$S-cysteine. The majority of the radioactive material was in the injection peak (material which did not bind to the column), which comprised between 59-83% of the radioactivity in the serum samples over the first 8hrs. This material was possibly sulphate. The remaining two radioactive peaks, which eluted at approximately 16.5% (S16) and 21.5% (S18) of the gradient contained between 1-9% and 2-10% of the radioactivity in the serum sample respectively. These two $^{35}$S containing peaks S16 and S18 were identified as possible candidates for the felinine containing peptide and were therefore subjected to further analysis by amino acid analysis, mass spectrometry and amino acid sequencing.

Incorporation of $^{35}$S-cysteine into serum peptides over time - Figure 1 shows the incorporation of $^{35}$S-cysteine into fractions S16 and S18 over time. The level of incorporation of $^{35}$S into S16 was the greatest 1hr after the $^{35}$S-cysteine was administered, after which time it plateaued at approximately 62% of its maximum level. The level of incorporation of $^{35}$S into S18 was at a maximum 2hr after administration of the $^{35}$S-cysteine, dropping to approximately 35% of the maximum level 8hr after administration.

Comparison of the peak areas between cats of different gender (Experiment 2) - Following the identification of peaks S16 and S18 as possible candidates for a felinine containing peptide,
serum samples from cats of different gender were analysed to quantitate the level of these peptide fractions. Since plasma felinine levels in entire male cats are approximately 3-6 times higher than in either castrated males or females it was postulated that differences in the amounts of felinine containing peptide present in the serum of cats of different gender should also be observed.

Table I shows the peak areas of S16 and S18 in the serum, and also the calculated peptide concentration for S18 in the blood. The area of peak S16 was 1.4-2.3 times higher in entire male cats compared to castrated males, entire females and spayed females. Meanwhile, the area of peak S18, and consequently the amount of the peptide present, was 7-22 times higher in entire male cats compared to castrated males, entire females and spayed females. There were significant gender specific differences in the amounts of compounds in both peaks S16 (P<0.001) and S18 (P<0.0001), with entire males having significantly higher levels in their blood compared to cats of other gender. The quantity of peptide S18 present in cat serum has the strongest linkage to the gender of the cat.

Characterization of $^{35}$S-cysteine peptide fractions (Experiment 3) – The molar ratios of the amino acids found in fractions S16 and S18 as determined by amino acid analysis are shown in Table II. S16 showed the presence of Cys, Leu and Tyr; however analysis of the fraction without prior hydrolysis indicated the presence of both free Leu and Tyr in the sample. Consequently, based on the molar ratios of unhydrolysed and hydrolysed samples it is likely that all of the Tyr and at least some of the Leu found in the sample originated from free amino acids that are not in peptide form.
Hydrolysis of fraction S18 yielded the amino acids Glu, Gly and Cys in approximately equimolar amounts (Table II), and no free amino acids were found to be present. This stoichiometry is the same as seen in the tripeptide glutathione, which has the amino acid sequence \(\gamma\)-GluCysGly. Evidence that this peptide was not glutathione comes from the observation that identical acid hydrolysis of glutathione undertaken at the same time as that of S16 and S18, resulted in a different stoichiometry (1:1:0.007 for Glu, Gly and Cys respectively (Table II)). The majority of the Cys present in glutathione was destroyed under the hydrolysis conditions used. Further evidence that S18 is not glutathione comes from the fact that HPLC analysis of both oxidised and reduced forms of glutathione showed that neither co-elute from the HPLC column in a position similar to S18.

A possible explanation for the high recovery of Cys, following acid hydrolysis of fraction S18 is that the Cys is actually derived from felinine, and Cys itself does not occur in the S18 peptide. It has been found that free felinine is totally degraded during acid hydrolysis under the conditions used in our laboratory (18). However, Westall (3) found that during acid hydrolysis felinine degraded to cysteine and cystine, while Tallan et al. (5) reported that felinine degraded to cystine and cysteic acid, thus indicating a possible degradation pathway of felinine via Cys. Similarly Cys is normally partially degraded under acid hydrolysis conditions. In the case of S18 it is possible that felinine is more stable to acid hydrolysis when it is present within a peptide, resulting in slower degradation to Cys, which in turn is more stable to hydrolysis since it faces a shorter exposure time to acid. Therefore the level of Cys detected as a result of the destruction of the felinine containing peptide may be far greater than if the peptide contained Cys.
Mass Spectral Analysis of S18 - Mass spectral analysis of S18 resulted in a monoisotopic mass of 394 being detected (Figure 2). This is consistent with the presence of a protonated species of peptide containing Glu, Felinine, and Gly. Once the monoisotopic mass and charge state was determined this species (m/z 394) was selected and fragmented. This resulted in the production of collision induced dissociation (CID) MS/MS spectra, which displayed sequence ions at 319, 307.9, 264.9, 247.7, 178.9 and 161.9 (Figure 3A).

The molecular ion at m/z 319 corresponds to Glu-Felinine and represents the loss of a C-terminal Gly residue (M, 75); the strong molecular ion at 264.9 corresponds to Felinine-Gly indicating the loss of an N-terminal Glu residue (M, 129), and in this case most likely a γ-Glu residue as the cleavage of the γ-glutamyl bond which is favoured during CID (19). The m/z at 307.9 corresponds to protonated GSH, indicating the loss of the 2-methyl butanol from the felinine side chain. The remaining molecular ions: m/z 247.7, 178.9 and 161.9 correspond to variations of the Felinine-Gly sequence with either the additional loss of an OH group, the loss of the 2-methyl butanol from the felinine side chain, or both. MS/MS/MS performed on the most abundant ion (m/z 264.9) confirmed that the other ions in the MS/MS spectra (m/z 247.8, 178.9, and 161.9) were derived from it (Figure 3B).

The mass spectral data indicate that the sequence of the peptide S18 is γ-glutamylfelinylglycine.
DISCUSSION

This study has shown that intraperitoneal administration of $^{35}$S-cysteine to an entire male cat results in the incorporation of the $^{35}$S moiety into material found in 3 serum peaks separated by reversed phase HPLC; the injection peak, S16 and S18. The injection peak was not characterised but this is most likely composed of sulphate. Results from an experiment investigating S16 and S18 peaks in cats of different gender showed that although the amounts of material eluting under these peaks were gender dependent, the level of S18 was more strongly linked to the gender of the cat, with entire male cats expressing 7-22 times more S18 material than cats of other gender. Results from the amino acid analysis of S18 showed the presence of Glu, Gly and Cys, in a stoichiometry of approximately 1:1:1. The knowledge that felinine breaks down under conditions of normal acid hydrolysis and potentially could give rise to Cys, due to the loss of the 2-methyl butanol group, along with the fact that peak S18 did not co-elute with either GSH or GSSG, suggested that this peak was not glutathione. This information taken together with the clear linkage between the level of expression of S18 and the gender of the cat strongly indicated that S18 was more likely to contain a felinine containing peptide than S16. It was also noted that S18 when reconstituted following freeze-drying emitted a catty odour typical of stored felinine containing samples (unpublished observation). S18 and not S16, was therefore further characterised by mass spectral analysis and amino acid sequencing.

It is more likely that felinine is produced as the result of a glutathione conjugation reaction rather than de novo synthesis of the tripeptide, as no free felinine can be detected in any tissues or blood of the cat (7). Consequently, the $^{35}$S label present in the felinine containing peptide must originate from $^{35}$S-GSH. However we did not detect any $^{35}$S-GSH in the serum of the cat. This is
not altogether unexpected since firstly, although the level of GSH in the blood of most species is in the order of 1.3mM or lower (20), most is confined to the erythrocytes, with the plasma only containing between 1-30µM GSH (21). Secondly, the apparent level of GSH in most tissues and physiological fluids can alter rapidly post-mortem or post-collection. In particular plasma GSH levels can drop rapidly after collection; in fact mouse plasma has a $t_{1/2}$ for GSH of around 10min (20). Therefore it is unlikely, under the conditions used here, that the GSH present in the cat serum at the time of analysis would have been detectable.

Collision induced dissociation of MH+ parent ion species has been found to be a very effective means of determining the structures of glutathione conjugates. CID has been found to specifically favour the rupture of peptide bonds in glutathione conjugates, in particular the $\gamma$-glutamyl linkage (19). In fact MS/MS has been employed as a screening process for the detection of unknown glutathione conjugates by either searching for species which differ in molecular weight by 129, the result of the loss of the $\gamma$-Glu or scanning for a molecular ion of 308, which indicates those MH$^+$ ions which fragment to give protonated GSH (19). The fragmentation pathway of S18 seen in our work is typical of that seen with other glutathione conjugates following MS/MS (19, 22). Sequencing of S18 by mass spectrometry clearly indicates that the sequence of the peptide is Glu-Felinine-Gly, and the high relative abundance of the m/z 265 species is indicative that the residue removed is most likely a $\gamma$-Glu. Therefore based on the mass spectrometry data, the sequence of the felinine containing peptide isolated from cat serum is $\gamma$-glutamylfelinylglycine (Figure 4).

The biological role of glutathione (GSH) in protecting cells against chemically reactive and cytotoxic agents is well known. This protective function is due to the presence of the nucleophilic sulfhydryl group through which glutathione is able to form conjugates with reactive
compounds. The conjugates may then be excreted into the bile or eliminated via the mercapturic acid pathway into the urine. The formation of GSH conjugates has been shown to be mediated either by specific GSH transferases in the liver, or to be nonenzymatic in nature (23).

In the case of the formation of \(\gamma\)-glutamylfelinylglycine, both of the substrates GSH and IPP are naturally found in all mammals, therefore, if the reaction was nonenzymatic in nature then felinine could be expected in the urine of species other than the Felidae. It is therefore most likely that an enzyme specific to selected cat species and under hormonal control is responsible for the formation of \(\gamma\)-glutamylfelinylglycine. The liver is a prime candidate for the site of \(\gamma\)-glutamylfelinylglycine synthesis because of its high metabolic capacity as well as being a major site of cholesterol synthesis and also the largest supplier of glutathione in the body. The \(\gamma\)-glutamylfelinylglycine would then be transported from the liver via the blood stream to the kidney where the enzymes responsible for the degradation of GSH; \(\gamma\)-glutamyltransferase and aminopeptidase M, could degrade the \(\gamma\)-glutamylfelinylglycine into its constituent amino acids. The Glu and Gly could be recycled and the felinine would be excreted in the urine. As free felinine would not be present in any organs or the blood, this would explain the observations by several researchers that free felinine has only been found to any great extent in the urine of cats, and trace amounts in the kidney, where we propose felinine is liberated from the \(\gamma\)-glutamylfelinylglycine peptide, and in the bladder, where contamination from the urine is clearly possible. However, this hypothesis remains to be proven.

It can be calculated from the average glomerular filtration rate (2.94ml/min/kg), the renal blood flow (10.61ml/min/kg) and the 24hr excretion rate of felinine by entire male cats (1, 24), that the minimum concentration of the felinine containing peptide in the blood of entire male cats assuming 100% clearance efficiency by the kidneys is 41.5nmol/ml. The concentration of
tripeptide in the blood of the entire male cats in the present study was determined to be 80.6nmol/ml (Table I). The latter value is in the same order of magnitude and sufficient to account for the levels of felinine found in the urine of entire male cats.

It is unlikely that the biological reason for the formation of \( \gamma \)-glutamylfelinylglycine in cats is one of cell protection by the conjugation of potentially damaging agents given that all mammals contain the substrates required for \( \gamma \)-glutamylfelinylglycine synthesis. The fact that the levels of both felinine and \( \gamma \)-glutamylfelinylglycine produced by cats are so clearly gender linked, along with the fact that females, or castrated males can be induced to produce felinine following testosterone injections (25), adds further weight to the suggestion that the biological role of felinine is as a precursor to a pheromone.

We have conclusively shown that contrary to all other reports, felinine can be found in the blood of cats albeit as part of a larger molecule. This discovery raises major questions regarding the validity of the current theory, which has prevailed for the past 50 years; that felinine is synthesised in the kidney and provides for the possibility that felinine may be synthesised in tissues other than the kidney.
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Footnote

1 The abbreviations used are: IPP, isopentenylpyrophosphate; GSH, glutathione; CID, collision-induced dissociation.
Legends

Fig. 1. Incorporation of $^{35}$S radioactivity into fractions S16 (?) and S18 (?) over time.

Fig. 2. Nanospray mass spectrometry spectrum of fraction S18.

Fig. 3. Structural characterization of fraction S18 by nanospray mass spectrometry. The CID spectrum of m/z 394, and the MS/MS fragmentation patterns are shown in 3A, the CID spectrum of m/z 264.89 and MS/MS/MS fragmentation patterns are shown in 3B.

Fig. 4. Structure of $\gamma$-glutamylfelinylglycine.
Table I

Peak area (x1000) for the two sulphur-containing compounds in blood serum and the peptide concentration in the blood of cats according to gender.

| Gender        | n  | Area Peak S16  | Area Peak S18  | Peptide Conc. (nmol/ml blood)† |
|---------------|----|----------------|----------------|--------------------------------|
| Entire male   | 5  | 6661 ± 377a    | 1799 ± 260a    | 80.6 ± 11.7                    |
| Castrated male| 6  | 4298 ± 497b    | 255 ± 26b      | 11.4 ± 1.2                     |
| Entire female | 7  | 4616 ± 280b    | 214 ± 43b      | 9.6 ± 1.9                      |
| Spayed female | 4  | 2927 ± 814b    | 82 ± 20b       | 3.7 ± 0.9                      |

ANOVA F(4,18), p< 0.001 0.0001 0.0001

Values are presented as mean ± SEM.

Values with different superscripts within columns were significantly different (P<0.05).

† Determined from the amino acid content of peak S18 after isolation using reversed-phase HPLC.
Table II

Molar ratio of amino acids in S16 and S18 and GSH as determined by amino acid analysis.

|       | Molar ratio |            |            |
|-------|-------------|------------|------------|
|       | Peak S16    | Peak S18   | GSH        |
| Glu   | -           | 1.07       | 1.03       |
| Gly   | -           | 1.00       | 1.00       |
| Cys   | 0.42        | 0.83       | 0.007      |
| Leu   | 3.18        | -          | -          |
| Tyr   | 1.00        | -          | -          |
Figure 1

Graph showing changes in \( ^{35}S \)-radioactivity (cpm/ml) over time (h). The graph displays two lines, one represented by filled circles and the other by open circles, indicating a peak at 2 hours followed by a decline.

\( ^{35}S \)-radioactivity (cpm/ml) vs Time (h)

0 1000 2000 3000 4000 5000 6000

0 2 4 6 8
**Figure 2**
Figure 3A
Figure 4
Isolation and characterization of a felinine containing peptide from the blood of the
domestic cat (felis catus)

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