Journal of Chromatography, 321 (1985) 421–432
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,459

RAPID HIGH-YIELD PURIFICATION OF CANINE INTESTINAL MOTILIN AND ITS COMPLETE SEQUENCE DETERMINATION

J. R. REEVE, Jr.*, F. J. HO and J. H. WALSH
Center for Ulcer Research and Education, Wadsworth Veterans Administration Medical Center, Los Angeles, CA 90073 (U.S.A.)
and
C. M. BEN-AVRAM and J. E. SHIVELY
Beckman Research Institute of City of Hope, Duarte, CA 91010 (U.S.A.)
(Received November 27th, 1984)

SUMMARY

Canine motilin has been purified from small amounts of canine intestine in a form suitable for microsequence analysis. The sequence determined is: Phe-Val-Pro-Ile-Phe-Thr-His-Ser-Glu-Leu-Gln-Lys-Ile-Arg-Glu-Lys-Glu-Arg-Asn-Lys-Ile-Arg-Asn-Lys-Gly-Gln. Canine motilin differs from porcine motilin at five positions. The rapid, high-yield (24% overall yield) microisolation techniques used for canine motilin should be suitable for the isolation of other basic peptides found in low levels in tissue that is available only in limited amounts. These methods should make the isolation and sequence determination of human brain and gut peptides more readily achievable.

INTRODUCTION

Motilin was first isolated and sequenced from porcine intestine, and shown to be a peptide containing 22 amino acids[1]. It has been more recently isolated in our laboratories from canine intestine[2], but a unique amino terminus could not be determined, and the amino acid composition did not agree with the sequence determined. These problems arose from the fact that motilin was contaminated either with free amino acids (found in the buffers or in the glassware used), or with other peptides that coeluted in the rather broad motilin peak.

The first isolation attempt produced less than optimal recovery. Less than 3% of the original immunoreactivity was recovered in the final step. Brain and gut peptides have been typically isolated with less than 10% recovery. The low recoveries coupled with limited amounts of available tissue have prevented structural characterization of most brain and gut peptides from species other than those used commercially.

New methods of mRNA characterization have made it possible to determine the sequence of the precursor of some of the brain and gut peptides from limited

0021-9673/85/$03.30

amounts of tissue[3–8]. However, these methods are not suitable for determining the processed peptides that are stored in the tissue. Microisolation and microsequence analysis must be utilized if the processed tissue forms of peptides are to be characterized.

These techniques have led to the elucidation of previously unknown processing sites for gastrin-releasing peptide[9], gastrin[10,11] and cholecystokinin[12] from gut tissue that could not have been predicted by cDNA techniques. They have also been used to compare the forms in various organs or normal tissue forms *versus* tumor tissue forms. Canine brain cholecystokinin has been shown to be identical to canine intestinal cholecystokinin[13]. Human tumor vasoactive intestinal peptide (VIP) has been shown to be identical to normal human intestinal VIP[14], and human tumor gastrin releasing decapeptide has been shown to be identical to canine intestinal gastrin-releasing decapeptide[15]. For all of these important findings good recovery was essential for structural identification. The techniques learned during these isolations were used for motilin and are described as an example of microisolation techniques for basic peptides that are suitable for subsequent microsequence analysis.

EXPERIMENTAL

Equipment

An Altex (Berkeley, CA, U.S.A.) Model 312 MP liquid chromatograph equipped with a 210 sample injector and an Altex–Hitachi 155-40 detector for 220 nm, and a LDC UV III monitor for 280 nm were employed for high-performance liquid chromatography (HPLC). Separations were performed on Waters (Milford, MA, U.S.A.) C_{18} and alkyl phenyl Z module reversed-phase columns (Waters μBondapak 10 μm, 10 cm × 8 mm) and a Vydac C_{18} reversed-phase column (Separation Group, 5 μm 25 cm × 4.6 mm).

Reagents and chemicals

Ammonium acetate was from Mallinckrodt (St. Louis, MO, U.S.A.), HPLC-grade water and acetonitrile were from Baker (Phillipsburg, NJ, U.S.A.). Trifluoroacetic acid was from Sigma (St. Louis, MO, U.S.A.) and was distilled from alumina then ninhydrin before use. CM-Cellulose was from Whatman (Clifton, NJ, U.S.A.). Sephadex G-50 was from Pharmacia (Piscataway, NJ, U.S.A.). Sep-Pak C_{18} cartridges were from Waters. Synthetic porcine and canine motilin were purchased from Peninsula Labs. (San Carlos, CA, U.S.A.).

Radioimmunoassay

Canine motilin was detected by a radioimmunoassay using an antibody (7921) raised to procine motilin. Synthetic porcine motilin (Peninsula Labs.) was used for standard and for label preparation. Label was prepared using a modified chlorine T method and the immunoassay was done as previously described[16] using an antibody dilution of 1:15,000.

Tissue extraction

Intestines from three dogs killed with Repose™ were obtained within 5 min after death. Luminal contents were rinsed with water and the unopened intestine was

boiled in water for 10 min. The upper one-third (duodenum and upper jejenum) mucosa was separated from muscle by scraping. Mucosa was then frozen at −40°C until extraction.

Frozen tissue (200 g) was broken into small pieces and homogenized. in 2% trifluoroacetic acid (500 ml) in a polytron homogenizer. Another 500 ml of 2% trifluoroacetic acid was added and the mixture was stirred for 6 h at 2–4°C. The extract was centrifuged (4°C, 3000 *g* for 1 h) and the supernatant was titrated to pH 5 using dilute ammonium hydroxide. The precipitate that was formed during the pH adjustment was removed by centrifugation (4°C, 3000 *g* for 3 h).

Sep-Pak concentration

The supernatant was loaded in 50-ml pools onto Sep-Pak C_{18} cartridges using a syringe driven by a Harvard pump at a flow-rate of approximately 1 ml/min. After loading, the cartridges were washed with 20 ml of 0.1% trifluoroacetic acid and eluted with three aliquots of 50% acetonitrile containing 0.1% trifluoroacetic acid.

Sephadex G-50 chromatography

The volume of the 50% acetonitrile eluate from the Sep-Pak step was reduced by vacuum in a vacuum desiccator containing phosphorus pentoxide and sodium hydroxide. The concentrated solution was loaded onto a Sephadex G-50 column (180 × 5 cm) and eluted with 3% acetic acid at a flow-rate of 80 ml/h. The eluted fractions (20 ml) were measured for motilin immunoreactivity and absorbance at 280 nm.

CM-Cellulose chromatography

The G-50 fractions containing the motilin immunoreactivity were pooled and applied to a CM-cellulose column (30 × 1 cm) equilibrated in 0.1 *M* ammonium acetate, pH 5.0. After loading, the column was rinsed with 200 ml of the same buffer and eluted with a 400-ml linear gradient of ammonium acetate (0.1–1.0 *M*, pH 5) at a flow-rate of approximately 20 ml/h. Fractions (2 ml) were collected and measured for motilin immunoreactivity and absorbance at 280 nm.

Waters Z module C_{18} gradient HPLC

The CM-cellulose purified motilin immunoreactivity was loaded onto a Z module C_{18} reversed-phase column equilibrated with buffer A (0.1% trifluoroacetic acid). After loading, the column was rinsed with buffer A then eluted with increasing concentrations of buffer B (50% acetonitrile containing 0.1% trifluoroacetic acid). Buffer B was increased to 30% in 5 min, held there for 33 min, then increased to 50% with a linear gradient over 40 min, this percentage was maintained for 20 min and then increased to 100% with a linear gradient over 20 min. The column effluent was monitored at 220 and 280 nm. Fractions (2 ml) were collected and assayed for motilin immunoreactivity.

Waters Z module alkyl phenyl gradient HPLC

The C_{18} purified motilin immunoreactivity was diluted three-fold with buffer A and loaded onto an alkyl phenyl reversed-phase column. The rinsing and elution steps were essentially the same as those described above for the C_{18} reversed-phase step.

Vydac C_{18} gradient HPLC

The alkyl phenyl purified motilin immunoreactivity was diluted, loaded and eluted in the same manner as other HPLC steps. After the first Vydac C_{18} chromatography it appeared that there were still contaminating peptides, so the immunoreactive peak was diluted and chromatographed once again on the Vydac C_{18} column using similar procedures.

Analytical Vydac C_{18} isocratic HPLC

Approxcimately 150 pmol of natural canine motilin was injected and isocratically eluted with buffer A–buffer B (52:48). The same amount of synthetic motilin was separately chromatographed, then the natural and synthetic peptides were co-injected.

Amino acid analysis

Approximately 0.1 nmol of purified canine motilin from the final HPLC step was dried down and hydrolysed in 5.7 *M* hydrochloric acid and containing 0.02% 2-mercaptoethanol for 24 h at 110°C. Approximately 2.0 nmol of synthetic canine motilin was chromatographed under the same conditions as the natural motilin final purification. Approximately 0.2 nmol was dried and hydrolyzed in the same way as the natural peptide. Hydrolysates were applied to a Beckman 121MB analyzer as previously described[17].

Sequence analysis

A portion of the purified peptide (3 nmol) was subjected to automated Edman degradation on a modified Beckman 890 sequencer as previously described[18]. Phenylthiohydantoin derivatives of amino acids were analyzed on an Ultrasphere ODS column performed on a Waters system equipped with 254 and 313 nm detectors; peaks were integrated and gradient elution was controlled by a Spectra-Physics 4000 integrator system[19].

RESULTS

Purification of the motilin immunoreactivity

The purification steps for canine motilin from a limited amount (200 g) of canine upper intestinal mucosa are shown in Table I. The immunoreactivity compared to the total absorbance at 280 nm indicated that the motilin had been purified 18,000 fold in the seven chromatographic steps. At each step the recovery of immunoreactivity was greater than 80% if all the immunoreactivity was accounted for except for the first HPLC step, the recovery of which was 69%. Much of the loss of immunoreactivity was predictable, because only the fractions containing the highest amounts of motilin were pooled for further purification. Even with the losses caused by not pooling all of the immunoreactive fractions, 24% of the activity in the crude extract was recovered in the final step.

The crude extract was partially purified and concentrated from 2 l to approximately 40 ml by the Waters Sep-Pak chromatography. Acetonitrile concentrations were lowered and the volume further reduced by putting the solution in a vacuum desiccator over phosphorus pentoxide. Little immunoreactivity was lost during either

TABLE I

CANINE MOTILIN PURIFICATION

Step	*Total absorbance (A_{280} × volume)*	*Amount RIA (nmol)*	*Amount RIA* / total absorbance*	*Purification (times)*	*Step recovery (%)*	*Total recovery (%)*
Crude extract	45,000	30.7	0.0007	1	100	100
Sep-Pak	2,925	25.6	0.009	13	83	83
Sephadex G-50	85	27.4	0.32	470	107	89
CM-Cellulose	32	26.7	0.83	1,200	97	87
Z Module C_{18}	6.0	12.9**	2.1	3,100	48***	42
Z Module phenyl	3.0	11.1	3.7	5,400	86	36
Vydac C_{18}	0.72	7.4	10.2	15,000	67§	24
Vydac C_{18}	0.63	7.5	11.9	18,000	101	24

* The ratio of radioummunoassay (RIA) amount (nmol)/total absorbance (absorbance at 280 nm × volume) is 12.5 for synthetic porcine motilin.

** Two fractions were separately purified after this step. The purified peptides were identical during isocratic and amino acid analysis and subsequent recoveries are based on the total in the two fractions.

*** Only 12.9 of 18.1 nmol were purified further. Step recovery based on 18.1 nmol is 69%.

§ Low recovery due to 3.1 nmol left in side fractions and not purified further. Step recovery actually is 95% if all immunoreactivity is used in the calculation.

of these concentration steps. The concentrated pool (23 ml) was loaded onto a Sephadex G-50 column and the motilin immunoreactivity eluted as a single peak on the shoulder of the major absorbance peak at 280 nm (Fig. 1A). Nearly 90% of the immunoreactivity was recovered after concentration and gel permeation chromatography.The pooled immunoreactivity from the gel permeation column was then further purified by ion-exchange chromatography. Motilin eluted as a single immunoreactive peak well separated from the major absorbance peaks (Fig. 1B). Nearly quantitative recovery was obtained and the immunoreactivity was purified 37-fold.

The greatest losses occurred during the first HPLC step. The total recovery at this step was 69%, but the motilin immunoreactivity eluted as three separate peaks (Fig. 2A). The major peak contained 12.9 nmol of motilin immunoreactivity which was 70% of the recovered immunoreactivity. The other peaks were not well defined and none contained more than 10% of the recovered immunoreactivity. The major peak was divided into two fractions and the rest of the figures show the purification of the first of these two fractions. However the second peak was purified using the same steps and Table I reflects the recovery of the material from both fractions. After the final purification motilin immunoreactivity from both pools co-eluted with synthetic canine motilin and had similar amino acid analysis.

Much better recoveries were obtained for the other HPLC steps with step recoveries of 90% or greater being obtained if all the immunoreactivity was accounted for. The alkyl phenyl step (Fig. 2B) resulted in one immunoreactive peak in the center of a rather broad absorbance peak. Only the fraction with the highest immunoreactivity was used for further purification. The next two HPLC steps were on a Vydac C_{18} analytical column. The first Vydac purification yielded two absorbance peaks with motilin immunoreactivity only associated with the later eluting peak (Fig. 3A). This material was diluted with buffer A and reloaded onto the same Vydac C_{18}

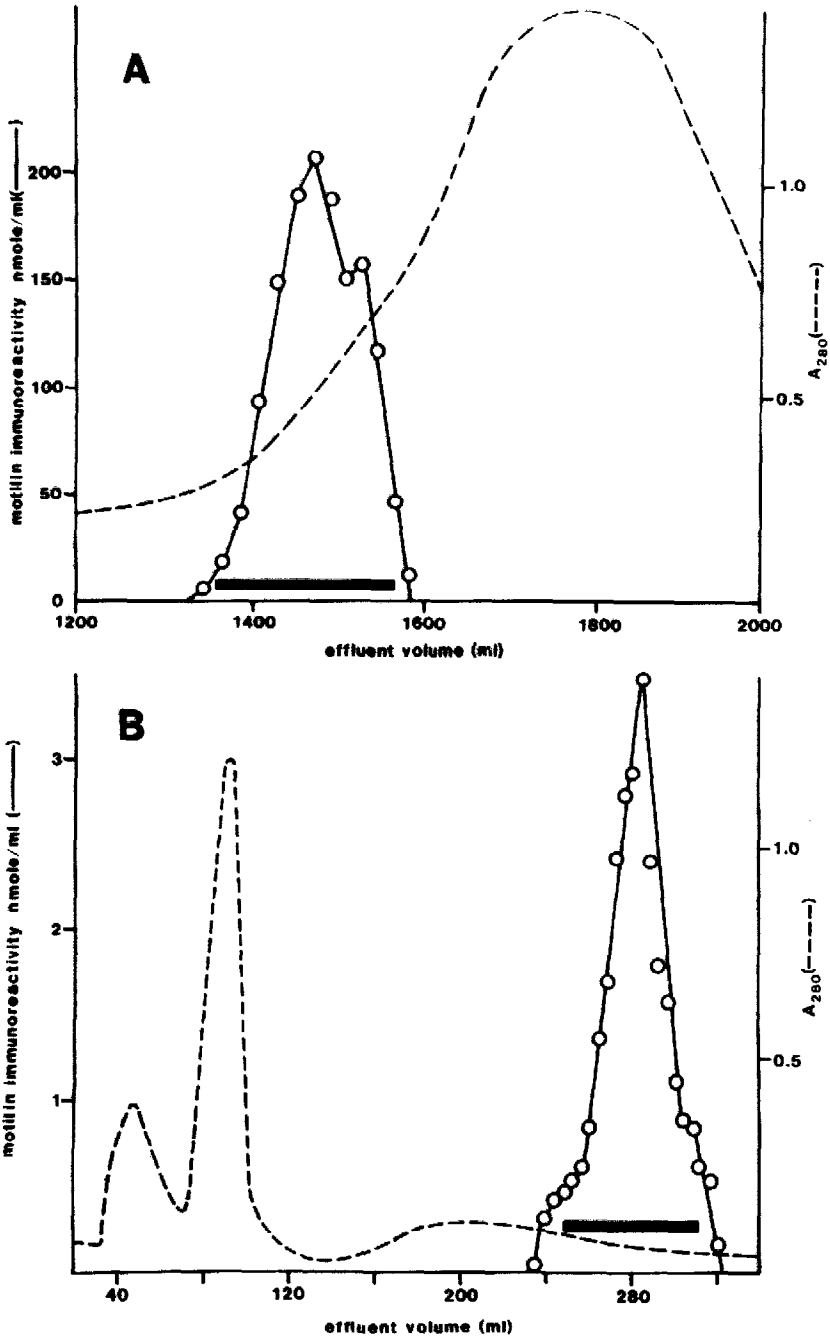

Fig. 1. (A) Elution profile of the Sep-Pak-concentrated motilin immunoreactivity on Sephadex G-50 SF (90 × 5 cm). The column was eluted with 3% acetic acid at a flow-rate of 80 ml/h and the fractions (20 ml) were measured for motilin immunoreactivity (———) and absorbance at 280 nm (– – –). Pooled fractions are shown by the solid bar. (B) Elution profile of the Sephadex G-50 purified motilin immunoreactivity on CM-cellulose. The pooled fractions from the Sephadex column were applied to a CM-cellulose column (30 × 1 cm) equilibrated in 0.1 *M* ammonium acetate pH 5.0. Elution was done as described in text and fractions (2 ml) were measured for motilin immunoreactivity (———) and absorbance at 280 nm (---------). Pooled fractions are shown by the solid bar.

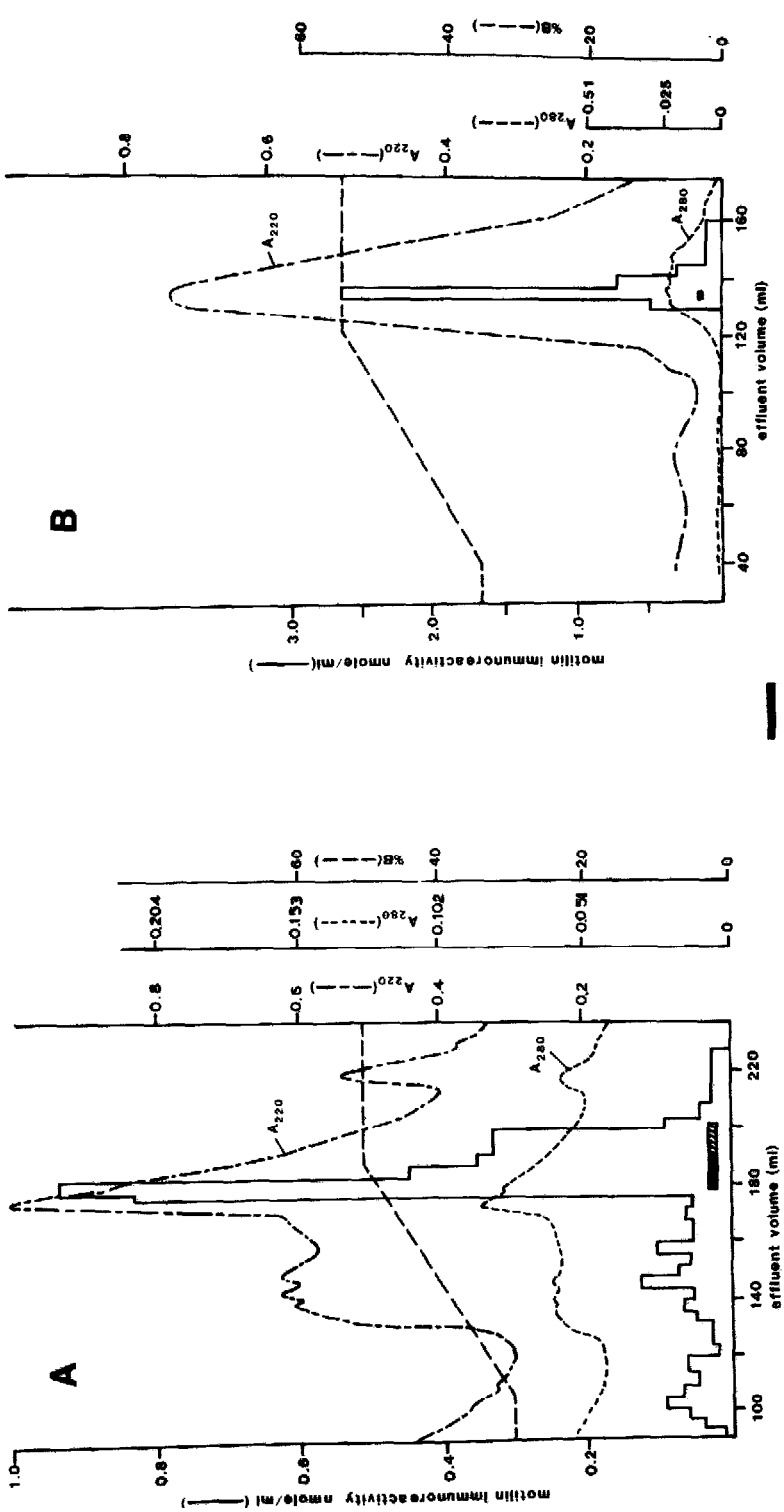

Fig. 2. (A) Elution profile of the CM-cellulose purified motilin immunoreactivity by Waters Z module C_{18} reversed-phase HPLC. Loading and elution are described in the text. Fractions (2 ml) were measured for motilin immunoreactivity (———). Absorbances at 220 nm (– · · –), and at 280 nm (– – – –) were measured by flow-through detectors. Two pools were purified separately, the earliest eluting pool (solid bar) was used for microsequence analysis data reported. The second pool (hatched bar) was also purified and characterized. It co-eluted during isocratic HPLC and had the same amino acid analysis as the material in the first pool. Overall recoveries shown in Table I are based on the purification of both fractions. (B) Elution of the Waters C_{18} purified motilin immunoreactivity by Waters alkyl phenyl reversed-phase HPLC. Loading and elution are described in the text. Fractions (2 ml) were measured for motilin immunoreactivity (———). Absorbance at 220 nm (– · · –) and 280 nm (– – – –) were measured by flow-through detectors. The solid bar indicates the fraction used for further purification.

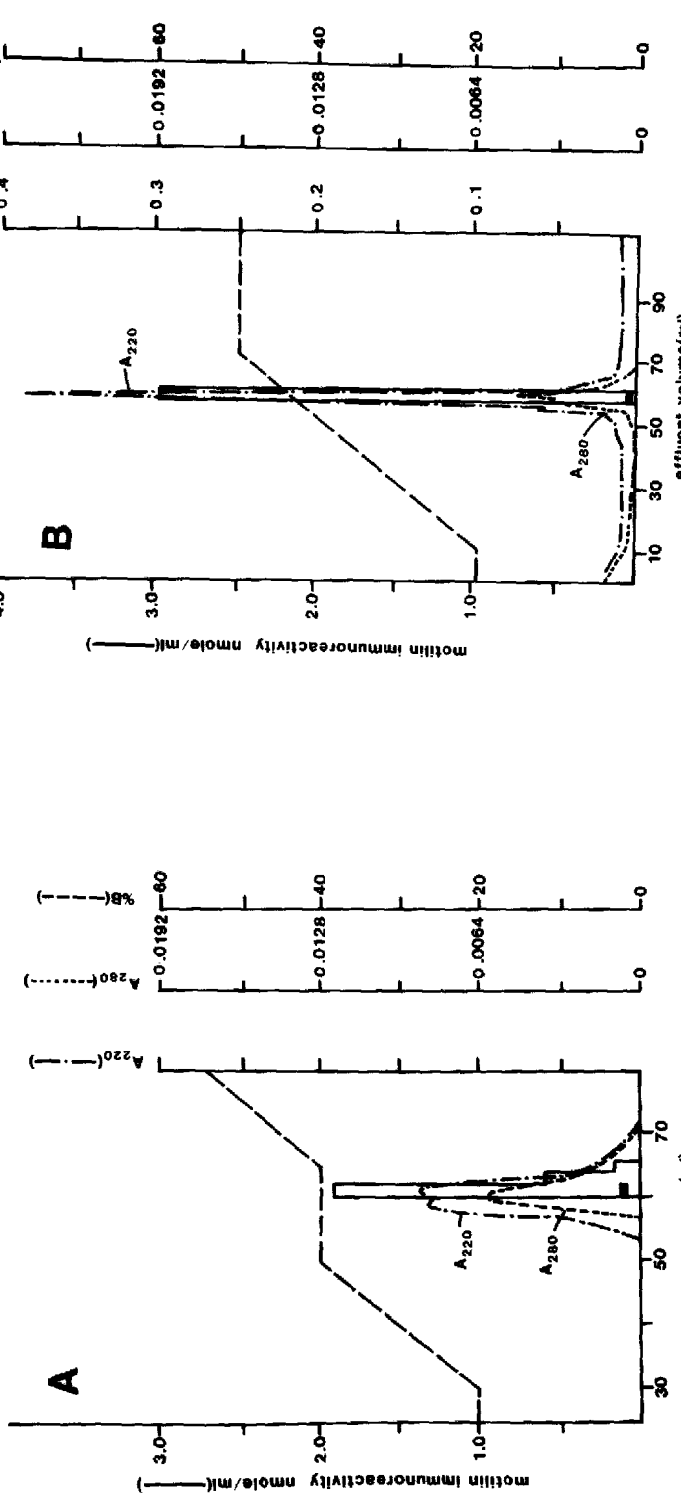

Fig. 3. (A) The elution of the alkyl phenyl purified motilin immunoreactivity by Vydac C_{18} reversed-phase HPLC. Loading and elution are described in the text. Fractions (2 ml) were measured for motilin immunoreactivity (———). Absorbances at 220 nm (— · —) and 280 nm (— — —) were measured by flow-through detectors. The solid bar indicates the fraction used for further purification. (B) The motilin from the first Vydac C_{18} was chromatographed a second time on the same column. Fractions were collected so single absorbance peaks were pooled then measured for motilin immunoreactivity (———). Absorbances at 220 nm and 280 nm were measured by flow-through detectors. The fraction used for amino acid and microsequence analysis is shown by the solid bar.

column. A single absorbance peak was associated with the eluted immunoreactivity (Fig. 3B) and this material was used for amino acid analysis, microsequence analysis and co-chromatography studies.

Analytical elution of natural and synthetic canine motilins

The elution profiles of the analytical isocratic HPLC of natural and synthetic motilins are shown in Fig. 4. First natural motilin was injected and the absorbance at 220 nm observed. Next synthetic motilin was chromatographed and it was noted how much injection volume would be required to match the absorbance of the natural material (results not shown). This amount was then injected and a peak size that was similar to the natural motilins peak size was observed. Finally the same amounts of motilin that were injected for the natural and synthetic motilins were mixed and injected onto the chromatograph. A single peak that was twice the size of the individual peaks revealed that the natural and synthetic peptides were co-eluting.

Amino acid analysis

The amino acid analysis of approximately 100 pmol of natural canine motilin is compared to the analysis of approximately 250 pmol of the synthetic peptide in

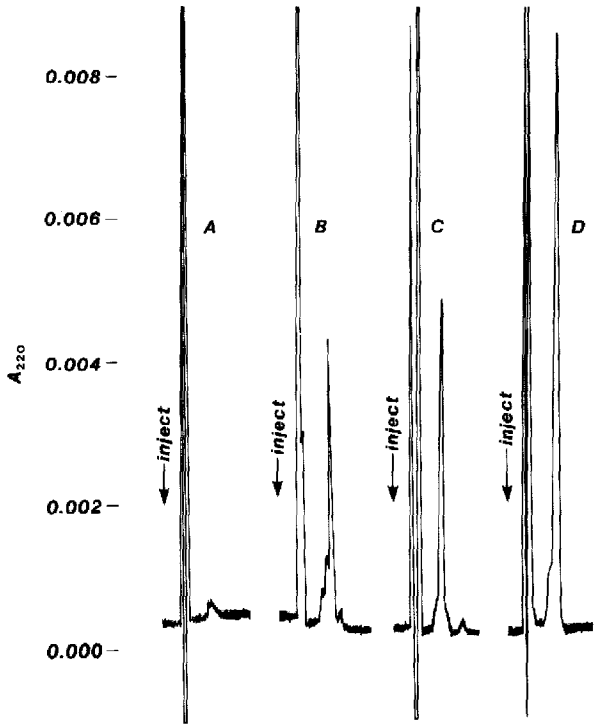

Fig. 4. Isocratic elution of synthetic and natural canine motilin. (A) Buffer blank. (B) Natural canine motilin. (C) Synthetic motilin. The volume of injection for synthetic motilin was estimated so its absorbance would be approximately the same as the natural canine motilin in panel B. (D) Co-injection of natural and synthetic motilins. The same volumes of injections as used in B and C were mixed then injected together.

Table II. The compositions were nearly identical and close to the values expected from sequence analysis.

Microsequence analysis

Approximately 3 nmol of canine motilin was used for the microsequence analysis. The initial yield at the amino terminus was 33% which is typical for basic peptides of this length. The results of the analysis of the phenylthiohydantion derivatives are shown in Table III. A unique derivative was obtained at each step and the sequence determination was possible for the entire sequence.

DISCUSSION

We have isolated canine motilin in amounts suitable for microsequence analysis from 200 g of intestinal mucosa, and yields were enough that the study could have been done on the mucosa obtained from a single dog intestine. These results are important for several reasons. First, it demonstrates that sequence data can be obtained from single individuals which should make the isolation and structural analysis of the human gut peptides feasible. The methods described here should be applicable for other basic peptides found in low amounts, where tissue amounts are limited. The most important changes of this method over the previously reported method[2] are: (1) Sep-Paks were used instead of a CM-cellulose batchwise procedure to concentrate the initial extract, resulting in an 83% yield instead of a 62% yield. (2) The gel filtration was done at acid pH instead of the basic pH used in the previous study resulting in an 89% recovery instead of a 60% recovery. (3) Motilin-containing

TABLE II

AMINO ACID ANALYSIS OF CANINE MOTILIN

Residue	*Mole%*	*Natural peptide* (residues/molecule)***	*Mole%*	*Synthetic peptide* (residues/molecule)***	*Sequence composition*
Asx	5.7	1.3	5.0	1.1	1
Thr	4.0	0.9	4.2	0.9	1
Ser	6.6	1.5	5.5	1.2	1
Glx	21.9	4.9	24.6	5.5	5
Pro	6.5	1.4	4.0	0.9	1
Gly	7.1	1.6	6.5	1.4	1
Ala	2.0	0.4	0.4	0.1	0
Val	2.5	0.6	2.9	0.6	1
Met	0.0	0.0	0.0	0.0	0
Ile	4.4	1.0	6.1	1.4	1
Leu	5.4	1.2	5.0	1.1	1
Tyr	0.7	0.2	0.0	0.0	0
Phe	5.3	1.2	6.6	1.5	2
His	6.4	1.4	5.9	1.3	1
Lys	13.7	3.0	15.3	3.4	3
Arg	7.3	1.6	8.0	1.8	2

* The amount of peptide analysed of the natural canine motilin was 0.2 μg and for the synthetic canine motilin 0.5 μg.

** Assumed 4.5 mole%/residue.

TABLE III

SEQUENCE ANALYSIS OF CANINE MOTILIN

Cycle	*Residue*	*Yield (pmol)*	*Sequence differences*	
			First canine report (ref. 2)	*Porcine motilin*
1	Phe	391	Ser, Lys, Phe*	
2	Val	975		
3	Pro	346		
4	Ile	654		
5	Phe	529		
6	Thr	50**		
7	His	46		Tyr
8	Ser	148***		Gly
9	Glu	305		
10	Leu	510		
11	Gln	299		
12	Lys	537		Arg
13	Ile	340		Met
14	Arg	128		Gln
15	Glu	391		
16	Lys	367		
17	Glu	269		
18	Arg	128		
19	Asn	141		
20	Lys	287		
21	Gly	134		
22	Gln	66		

* In the first report on the structure of canine motilin[2] we were not able to clearly identify a unique amino terminus, but the other 21 cycles revealed the same sequence as we are now reporting.

** Phenylthiohydantoin-threonine yields three products, but the integration of only one is reported.

*** Phenylthiohydantoin-serine yields two products, but the integration of only one is reported.

solutions were never concentrated to dryness which caused 20–60% losses during the previous isolation. (4) The change in HPLC columns and the eluting buffers resulted in 1.5-ml elution volumes instead of the 5-ml volumes seen before, thus the peptide could be better resolved from contaminants. The contaminants could be from two sources; other peptides eluting in the region of motilin, and contaminants in the HPLC buffers. Both sources of contaminants could have caused the differences between sequence and amino acid analysis data in the original report. These changes in methods have resulted in an overall yield of 24% (instead of 3%) even though less tissue was used for the repurification.

There was never any indication that canine motilin existed in more than one molecular form during this isolation procedure. However, three molecular forms of motilin were indicated by the first HPLC separation in the previous report. The multiplicity of forms could be artifacts of the extraction conditions since the original extraction was with boiling acetic acid *versus* the cold trifluoroacetic extractions used in this report. It is quite possible that one or more of the glutamine or asparagine residues were deaminated during the boiling acetic acid step which could account for the multiplicity of forms and the elution position of the uncharacterized immuno-

reactive peptides. No large motilin-like immunoreactivity was observed in canine intestine as was found in extracts of rat brains[20].

The sequence data obtained for residues 2–22 in the first report have been confirmed, and for the first time the amino terminal residue has been uniquely identified. The confirmation of the data should be reassuring for investigators concerned about the analysis of peptides at such low levels. The two sequences were done in independent laboratories using different instruments for the Edman chemistry and for the identification of the phenylthiohydantoin derivatives, and the results were essentially identical. Canine motilin differs from its porcine counterpart in 5 of its 22 residues, making it one of the most variable peptide hormones characterized. The biological consequences of these changes should be studied although preliminary comparisons indicate similar biological activity for induction of premature activity fronts for canine and porcine motilins[2]. Confirmation of the structure for canine motilin is three-fold: (1) the microsequence analysis agrees with that previously reported, (2) amino acid analysis of both synthetic and natural motilin are consistent with the structure determined, and (3) the synthetic and natural peptides co-elute during HPLC.

ACKNOWLEDGEMENTS

This work has been supported by NIH grants AM 17328, AM 29842, AM 33155 and the Veterans Administration.

REFERENCES

1 J. C. Brown, M. A. Cook and J. R. Dryburgh, *Can. J. Biochem.*, 51 (1973) 533–537.
2 P. Poitras, J. R. Reeve, Jr., M. W. Hunkapiler and L. E. Hood, *Regul. Peptides*, 5 (1983) 197–208.
3 E. Boel, J. Vuust, F. Norris, A. Wind, J. F. Rehfeld and K. A. Marcker, *Proc. Nat. Acad. Sci. U.S.*, 80 (1983) 2866–2869.
4 O. J. Yoo, C. T. Powell and K. L. Agarwal, *Proc. Nat. Acad. Sci. U.S.*, 79 (1982) 1049–1053.
5 E. R. Spinel, W. W. Chin, J. Price, L. H. Rees, G. M. Besser and J. F. Habener, *Proc. Nat. Acad. Sc. U.S.*, 81 (1984) 5699–5703.
6 G. I. Bell, R. F. Santerre and G. T. Mullenbach, *Nature (London)*, 302 (1983) 547–549.
7 N. Itoh, K.-I. Obeta, N. Yanaihara and H. Okamoto, *Nature (London)*, 304 (1983) 547–549.
8 R. J. Deschenes, L. J. Lorenz, R. S. Haun, B. A. Loos, K. F. Collier and J. E. Dixon, *Proc. Nat. Acad. U.S.*, 81 (1984) 726–730.
9 J. R. Reeve, Jr., J. H. Walsh, P. Chew, B. Clark, D. Hawke and J. E. Shively, *J. Biol. Chem.*, 258 (1983) 5582–5588.
10 R. A. Gregory, G. J. Dockray, J. R. Reeve, Jr., J. E. Shively and C. Miller, *Peptides*, 4 (1983) 319–323.
11 J. R. Reeve, Jr., J. H. Walsh, R. K. Tompkins, D. Hawke and J. E. Shively, *Biochem. Biophys. Res. Commun.*, 123 (1984) 404–409.
12 V. E. Eysselein, J. R. Reeve, Jr., J. E. Shiveley, D. Hawke and J. H. Walsh, *Peptides*, 3 (1982) 687–691.
13 V. E. Eysselein, J. R. Reeve, Jr., J. E. Shiveley, C. Miller and J. H. Walsh, *Proc. Nat. Acad. Sci. U.S.*, 81 (1984) 6565–6568.
14 N. W. Bunnett, J. R. Reeve, Jr., R. Dimaline, J. E. Shively, D. Hawke and J. H. Walsh, *J. Clin. Endocrinol. Metab.*, 59 (1984) 1133–1137.
15 M. S. Orloff, J. R. Reeve, Jr., C. M. Ben-Avram, J. E. Shively and J. H. Walsh, *Peptides*, 5 (1984) 865–870.
16 P. Poitras, J. H. Steinbach, G. VanDeventer, C. F. Code and J. H. Walsh, *Amer. J. Physiol.*, 239 (1980) G215–G220.
17 U. Del Valle and J. E. Shively, *Anal. Biochem.*, 96 (1979) 77–83.
18 J. E. Shively, *Methods Enzymol.*, 79 (1981) 31–48.
19 D. Hawke, P.-M. Yuan and J. E. Shively, *Anal. Biochem.*, 120 (1982) 302–311.
20 T. L. O'Donohue, M. C. Beinfeld, W. Y. Chey, T. M. Chang, G. Nilaver, E. A. Zimmerman, H. Yajima, H. Adachi, M. Toth, R. P. McDevitt and D. M. Jacobowitz, *Peptides*, 2 (1981) 467–477.