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Determination of carnosine and other biogenic imidazoles in equine plasma by isocratic reversed-phase ion-pair high-performance liquid chromatography

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ABSTRACT

The isocratic reversed-phase ion-pair high-performance liquid chromatographic technique presented provides a sensitive, rapid and reproducible analytical method for the selective determination of carnosine and other biogenic imidazoles in equine plasma. Plasma was deproteinized with 5-sulphosalicylic acid and the compounds of interest were isolated by sorbent extraction on Bond Elut PRS cartridges. Recoveries were 97–105% and the lowest limits of detection were 58.3–80.1 n*M*. All compounds of interest were well resolved within a maximum retention time of 9.2 min. The mean equine plasma carnosine level determined by this method was 11.31 μM. Comparative determinations were made in canine and human plasma. Carnosine was not detected in human plasma. Concentrations of imidazole in canine plasma are reported here for the first time.

INTRODUCTION

The imidazole dipeptide carnosine (β-alanyl-L-histidine) occurs at high concentrations in equine skeletal muscle. Recent studies have demonstrated mean muscle carnosine concentrations of 108.6 mmol/kg dry muscle (d.m.) in the Thoroughbred horse [1] and 156.8 mmol/kg d.m. in the Quarterhorse [2] for the gluteus medius muscle. It has been accepted that carnosine performs a major role as a physiological H^+ buffer in anaerobically contracting muscle [3]. Carnosine is also believed to function as a neurotransmitter in the olfactory epithelium [4] and as an anti-oxidant in muscle and other tissues [5].

Early methods for the determination of carnosine were based upon thin-layer chromatography [6] or electrophoresis [7] with subsequent quantitation on an ion-exchange amino acid analyzer [8]. Such procedures, however, suffer from long analysis times, poor resolution and low sensitivity. The introduction of high-performance liquid chromatography (HPLC) has rectified some of these problems. Binary gradient elution in conjunction with either pre- or post-column derivatization now forms the basis of several HPLC methods for the measurement of carnosine and histidine levels in tissue samples [1,9–13].

Difficulties have been encountered in the application of previously published methods to the determination of carnosine in equine plasma owing to the low plasma content and tendency to co-elute with amino acids or other small peptides. Analysis times are typically 30–60 min as the complex chromatograms produced contain superfluous information.

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A specific HPLC method for the quantitation of carnosine and anserine (β-alanyl-L-1-methylhistidine) in rabbit serum [14] proved to be grossly inaccurate when applied to equine plasma.

The analytical method presented combines isocratic reversed-phase ion-pair HPLC with sorbent extraction to provide rapid, selective and sensitive detection of carnosine, histidine, anserine, and 1-methylhistidine in plasma with previously unobtained specificity.

EXPERIMENTAL

Instrumentation

The HPLC system comprised a Constametric 3000 pump (LDC Analytical, Stone, UK), a Rheodyne 7125 injector with a 200-μl sample loop (Cotati, CA, USA), an LC–UV variable wavelength ultraviolet spectrophotometric detector (Unicam, Cambridge, UK) and an LKB 6500 flat-bed potentiometric recorder (LKB Biochrom, South Croydon, UK). A Sepralyte ODS 40-μm sacrificial column (50 mm × 4.6 mm I.D.) (Analytichem International, Harbor City, CA, USA) and a Rheodyne in-line filter (0.5 μm pore size) were inserted between the pump and the injector.

Ultraviolet absorption spectra were recorded on a Unicam SP800 ultraviolet spectrophotometer (Unicam).

Materials

Ammonium dihydrogenphosphate [$NH_4H_2PO_4$], sodium pentanesulphonate, sodium heptanesulphonate, sodium octanesulphonate, sodium dodecylsulphate, L-carnosine, L-anserine, L-homocarnosine, L-histidine and L-1-methylhistidine were purchased from Sigma (Poole, UK). Acetonitrile Far UV (HPLC grade) and methanol (HPLC grade) were purchased from Romil (Loughborough, UK). Phosphoric acid (H_3PO_4), concentrated hydrochloric acid and 5-sulphosalicylic acid (SSA) were purchased from Aldrich (Gillingham, UK). All chemicals and reagents were analytical reagent grade unless specified otherwise. Water was purified by reverse osmosis and deionisation (Elgastat Spectrum RO1, Elga, UK).

Sample preparation

Blood samples were obtained by venepuncture and collected in 5-ml lithium heparin tubes. The blood samples were immediately centrifuged for 4 min at 2000 *g* and at 4°C. Plasma (1 ml) was withdrawn, deproteinized with 200 μl of 30% (w/v) SSA, vortex-mixed and centrifuged. The supernatant was withdrawn and stored at −85°C until sorbent extraction and analysis.

Sorbent extraction

Plasma SSA extracts (500 μl) were loaded onto 100-mg Bond Elut PRS (propylsulphonyl) extraction cartridges (Analytichem International) previously conditioned with methanol (4 ml) followed by 1.0 *M* H_3PO_4 (4 ml). Interfering substances were washed from the cartridges with 1.0 *M* H_3PO_4 (2 × 500 μl). The sorbent was air-dried and the isolates (carnosine and other imidazoles) were eluted with HPLC mobile phase (2 × 500 μl). Solvents at all stages were drawn through the cartridges at a flow-rate of 1 ml/min. A 200-μl aliquot was injected onto the column.

HPLC conditions

Chromatography was performed on a Hypersil ODS (150 mm × 4.6 mm I.D.) analytical column (Shandon Scientific, Runcorn, UK) protected by a Hypersil ODS (20 mm × 4.6 mm I.D.) guard column. The analytical column packing material had a particle size of 3 μm and the guard column packing material had a particle size of 5 μm.

The compounds of interest were eluted isocratically at ambient temperature using a mobile phase comprising an aqeous solution of 200 m*M* $NH_4H_2PO_4$ (23.006 g/l) and 100 m*M* sodium pentanesulphonate (17.42 g/l) adjusted to pH 2.0 with concentrated hydrochloric acid and containing 4% (v/v) acetonitrile. The mobile phase was filtered through 0.45-μm Millipore HVLP 047 filters and degassed by helium sparging prior to use and periodically throughout the day. The mobile phase was freshly prepared each day. The flow-rate was 0.8 ml/min. UV absorption was measured at 220 nm wih the detector sensitivity set for full-scale deflection at 0.32 absorbance units. Plasma concentrations were determined by com-

paring sample peak heights to those of external standards.

Standard preparation

Individual 10 m*M* stock standard solutions of carnosine, anserine, histidine and 1-methylhistidine were prepared by dissolving the required weight of each compound in 10 ml of HPLC-grade water. Working standard solutions for each compound were prepared over the concentration range 5–30 μM by dilution of stock standards with mobile phase. Both stock standard solutions and working standard solutions were stored at −20°C when not used.

Recovery study

Pooled equine plasma was spiked at 25 μM with a mixed standard containing 1-methylhistidine, histidine, anserine and carnosine. Overall extraction recoveries (n = 5) for the combined deproteinization and sorbent extraction stages were determined by comparing the chromatograms obtained from the spiked plasma extracts with those obtained from a mixed standard solution containing 25 μM 1-methylhistidine, histidine, anserine and carnosine. The concentration of these compounds in the plasma prior to spiking was determined and taken into account when calculating the overall recoveries.

Reproducibility study

Standards for the determination of the reproducibility of the analysis were prepared from pooled equine plasma extract which was spiked at 25 μM with 1-methylhistidine, histidine, anserine and carnosine. Repeated injections of the spiked extract (n = 5) were made on day 1, day 5 and day 10 to assess the day-to-day variation. Within-day variation was determined by repeated injections (n = 5) of the spiked extract on three occasions within day 1.

Detection limits

The lower limits of detection for the compounds of interest were determined by injecting individual standards of 1-methylhistidine, histidine, anserine and carnosine sufficient to elicit full-scale deflection on the recorder at a detector sensitivity setting of 0.01 absorbance units. The height of the smallest detectable peak was interpolated for a signal-to-noise ratio of 2:1: *ca.* 5 mm.

RESULTS AND DISCUSSION

HPLC conditions

Spectra over the UV range 190–450 nm were recorded for the imidazole compounds of interest. Their mean λ_{max} values occurred at a wavelength of 220 ± 5 nm (carnosine, λ_{max} = 223 nm) in 0.1 *M* ammonium dihydrogenphosphate (pH 2.0). The reference cell contained 0.1 *M* $NH_4H_2PO_4$. The UV spectra of several other amino acids were also recorded and exhibited λ_{max} values at slightly lower wavelengths (β-alanine, λ_{max} = 205 nm). At 220 nm the absorbance

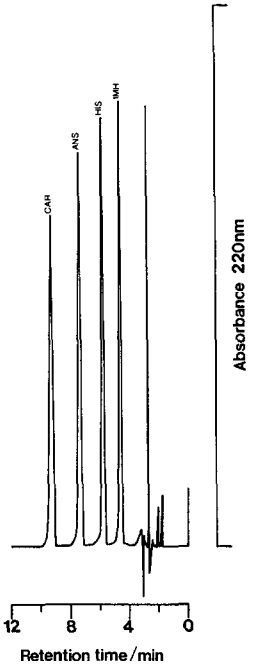

Fig. 1. Reversed-phase ion-pair HPLC separation of a mixed standard containing 25 μM 1-methylhistidine (1MH), histidine (HIS), anserine (ANS) and carnosine (CAR). Column: Hypersil ODS 3 μm (150 mm × 4.6 mm I.D.). Mobile phase: 200 m*M* $NH_4H_2PO_4$, 100 m*M* sodium pentanesulphonate, 4% (v/v) acetonitrile, pH 2.0. Flow-rate: 0.8 ml/min. Detector sensitivity: 0.32 absorbance units. Detector wavelength: 220 nm.

ratio of β-alanine to carnosine was 1.00:5.88 for equimolar solutions. A detection wavelength of 220 nm was selected.

Inevitably there is the potential for significant interference when utilizing UV detection at such a non-specific wavelength. In addition to the amino acids, many other polar low-molecular-mass compounds ($M_r < 2000$), endogenous to plasma, display strong UV absorption at 220 nm. Some such compounds are creatine, creatinine, purine nucleotides, nucleosides and bases, and carboxylates (lactate and pyruvate). It has, however, previously been demonstrated [15] that for a non-polar stationary phase and a polar mobile phase, retention of the biogenic imidazoles is maximised at low pH and furthermore, that creatine, creatinine and the purine-based compounds are minimally retained [16].

A variety of columns and mobile phase parameters were evaluated in order to optimize the resolution of the various imidazole compounds and to further minimize any potential interference. Aqueous potassium (or sodium) dihydrogenphosphate buffers have generally formed the basis of previous eluents [15,14] for the HPLC of carnosine and the other imidazoles. The use of alkali metal phosphates during the present method development caused considerable peak tailing of these basic compounds and was apparent in a previous method [15]. The alternative use of ammonium dihydrogenphosphate resulted in a marked improvement in peak symmetry. The effect of a range of ion-pairing agents (sodium pentanesulphonate, sodium heptanesulphonate, sodium octanesulphonate and sodium dodecylsulphate) and acetonitrile as the organic modifier on the retention and resolution of the compounds of interest was investigated. Sodium pentanesulphonate (100 m*M*) and 4% acetonitrile produced the best resolution within the optimum capacity factor (k') range: $1 < k' < 10$. Of the different analytical columns evaluated (Apex ODS1 5 μm, Apex Phenyl 5 μm, Spherisorb ODS2 5 μm, Hypersil ODS 5 μm and Hypersil ODS 3 μm) superior resolution was obtained with the latter. The resultant chromatographic conditions produced a good separation of 1-methylhistidine, histidine, anserine and carnosine as shown in Fig. 1. Carnosine and the other imidazoles eluted between 4.6 and 9.2 min. The retention times and capacity factors of the individual compounds are given in Table I. The efficiency of the separation is indicated by a resolution factor, $R_s > 4.2$ for all adjacent peaks.

Sample preparation and sorbent extraction

Deproteinization of plasma extracts using traditional reagents such as 1.0 *M* perchloric acid, 0.1% trifluoroacetic acid or methanol (three to four equivalent volumes) produce an unwanted dilution. This is particularly undesirable in the analysis of carnosine where the plasma concentration is known to be generally of the order of 10 μ*M*. The addition of a small volume (100 μl) of a concentrated solution of SSA efficiently precipitates plasma protein, minimises the dilution effect and is more reproducible than adding SSA in solid form.

Chromatography of SSA-deproteinized plasma was inadequate for the determination of carnosine as the carnosine peak eluted within the tailing side of a large front peak, thus making quantitation impossible at low levels. In addition, the peaks arising from 1-methylhistidine, histidine and anserine were entirely obscured by this large front peak. Furthermore, large peaks evolving from well retained compounds continued to appear in the chromatogram up to 22 min after sample injection and only after this time did the baseline absorbance begin a significant reduction towards the pre-injection value.

Sorbent (solid-phase) extraction was employed

TABLE I

RETENTION TIMES AND CAPACITY FACTORS (k') OF THE IMIDAZOLES

Compound	Retention time (min)	k'
1-Methylhistidine	4.6	1.56
Histidine	5.8	2.22
Anserine	7.4	3.11
Carnosine	9.2	4.11

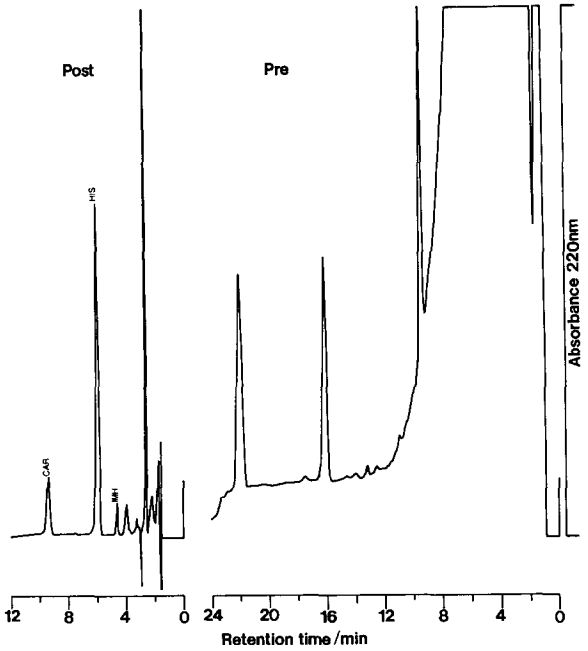

Fig. 2. Chromatograms of an SSA extract of equine plasma. (Pre) Before sorbent extraction. (Post) After sorbent extraction. HPLC conditions as in Fig. 1. Sorbent extraction details are given under Experimental.

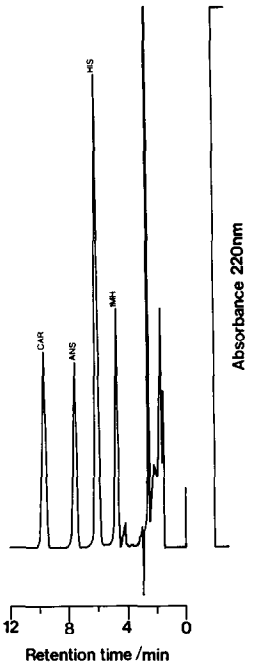

Fig. 3. Chromatogram of an equine plasma SSA extract spiked with an authentic mixed standard containing 1-methylhistidine (1MH), histidine (HIS), anserine (ANS) and carnosine (CAR). HPLC conditions as in Fig. 1.

to provide pre-chromatography sample clean-up. A variety of methodologies were investigated to determine the effectiveness of the technique. At low pH values, carnosine (and the other imidazole molecules) exist as cationic species. The degree of ionization of the imidazole ring and the terminal amine group approaches 100% at pH 2.0. This property was exploited by use of a cation-exchange mechanism for sorbent extraction. The requirement for an acid environment throughout the extraction procedure was simplified by the inherent acidic nature of the SSA-deproteinized plasma and by the requirement for an acidic final extract to provide good chromatography in the pH 2.0 mobile phase.

Using the sorbent extraction method described under Experimental an excellent purification of the deproteinized plasma was achieved, and few, if any, interference peaks were present in the final extract. The contrast in sample quality between pre- and post-sorbent extraction is demonstrated for a typical equine plasma extract in Fig. 2.

Spiking of a final equine plasma extract with a mixed standard, prepared from authentic compounds, produced only single peaks at the retention times for putatative carnosine and histidine endogenous to plasma and also at the retention times for 1-methylhistidine and anserine, as shown in Fig. 3. Peaks for anserine and 1-methylhistidine in the spiked extract were, however, absent in the normal final extract. Additionally, spiked canine plasma extracts displayed single peaks for each of the four imidazoles.

The recoveries (mean ± coefficient of variation, %) from spiked plasma by solid-phase extraction were: 1-methylhistidine, 103.7 ± 9.4%; histidine, 105.4 ± 9.9%; anserine, 97.0 ± 4.4%; carnosine, 101.8 ± 9.4%. The use of this deproteinization and extraction technique provides a highly selective determination of the biogenic plasma imidazoles.

TABLE II

WITHIN-DAY REPRODUCIBILITY OF THE DETERMINATION OF IMIDAZOLE COMPOUNDS IN SPIKED EQUINE PLASMA

Pooled final plasma extract was spiked at 25 μM for each individual compounds. Each value represents the mean ± S.D. for five determinations. C.V. = coefficient of variation.

Session	1-Methylhistidine		Histidine		Anserine		Carnosine	
	Concentration (μM)	C.V. (%)	Concentration (μM)	C.V. (%)	Concentration (μM)	C.V. (%)	Concentration (μM)	C.V. (%)
1	29.11 ± 1.80	6.18	67.96 ± 1.29	1.90	23.06 ± 1.88	8.15	31.96 ± 1.05	3.29
2	31.10 ± 1.49	4.79	71.49 ± 1.34	1.87	22.48 ± 1.65	7.34	33.29 ± 1.02	3.06
3	29.96 ± 1.70	5.67	70.42 ± 1.64	2.33	22.90 ± 1.84	8.03	32.50 ± 0.94	2.89
Mean	30.06 ± 1.67[a]	5.55	69.96 ± 1.43[a]	2.04	22.81 ± 1.79[a]	7.85	32.58 ± 1.00[a]	3.07

[a] Pooled within-session S.D.

Standards

The standard curves produced for carnosine and the other imidazole compounds showed a linear relationship between peak height and concentration in the range 5–30 μM. The linear regression equations for each compound were: 1-methylhistidine, $y = 6.21x + 0.61$ ($r > 0.99$); histidine, $y = 5.91x + 0.14$ ($r > 0.99$); anserine, $y = 5.48x + 0.54$ ($r > 0.99$); carnosine, $y = 4.65x + 0.11$ ($r > 0.99$) (y = peak height in mm and x = concentration in μM).

Reproducibility

Values for typical within-day and day-to-day variation are given in Tables II and III, respectively. In these tables, imidazole concentrations above 25 μM are a consequence of the endogenous plasma content. The within-day mean coefficients of variation were less than 7.9% for replicate determinations ($n = 5$) on three occasions for all compounds in spiked plasma. Day-to-day mean coefficients of variation were below 6.4% for all compounds when replicate determinations

TABLE III

DAY-TO-DAY REPRODUCIBILITY OF THE DETERMINATION OF IMIDAZOLE COMPOUNDS IN SPIKED EQUINE FINAL PLASMA EXTRACTS

The spiked concentrations were 25 μM. Each value represents the mean ± S.D. for five determinations. C.V. = coefficient of variation.

Day	1-Methylhistidine		Histidine		Anserine		Carnosine	
	Concentration (μM)	C.V. (%)	Concentration (μM)	C.V. (%)	Concentration (μM)	C.V. (%)	Concentration (μM)	C.V. (%)
Day 1	29.33 ± 2.41	8.22	74.43 ± 2.63	3.53	24.25 ± 1.09	4.49	31.16 ± 1.57	5.04
Day 5	31.12 ± 1.47	4.72	71.49 ± 1.34	1.87	22.48 ± 1.65	7.34	33.11 ± 1.35	4.08
Day 10	30.93 ± 1.83	5.29	66.95 ± 5.79	8.65	24.01 ± 1.45	6.04	29.47 ± 1.13	3.83
Mean	30.46 ± 1.94[a]	6.37	70.96 ± 3.75[a]	5.28	23.61 ± 1.42[a]	6.01	31.25 ± 1.36[a]	4.35

[a] Pooled day-to-day S.D.

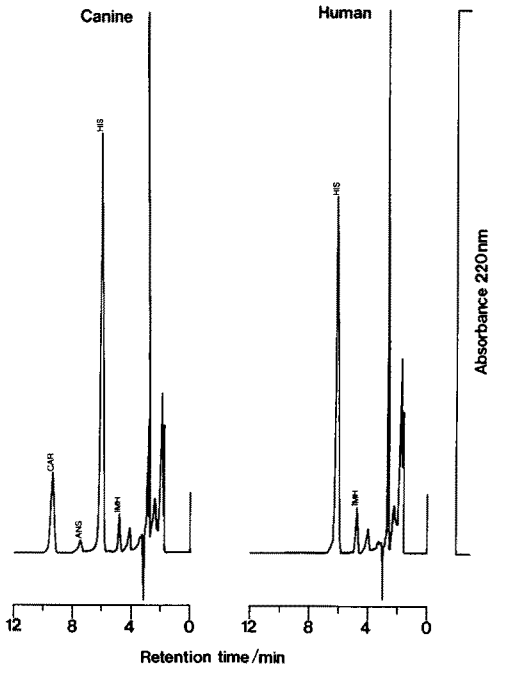

Fig. 4. Typical chromatograms of SSA extracts after sorbent extraction of canine plasma and human plasma. HPLC conditions as in Fig. 1. Detector setting for the human plasma sample was 0.64 absorbance units.

(n = 5) were made over a ten-day period. The method described displayed good reproducibility both within-day and on a day-to-day basis.

Detection limits

The lower limits of detection for 1-methylhistidine, histidine, anserine and carnosine were 58.3, 65.9, 71.5 and 80.1 nM, respectively.

Application to plasma samples

The current method was applied to the determination of carnosine and other endogenous imidazole compounds in normal equine, canine and human plasma samples. Typical chromatograms of canine and human plasma extracts are shown in Fig. 4. The plasma concentrations of carnosine and the other biogenic imidazoles for horse, dog and man, expressed as the mean ± standard deviation, are given in Table IV.

All the values for equine and human plasma, generated by the present method, are in good agreement with previously published data. The authors are unaware of any previously published data regarding canine plasma imidazole concentrations.

The equine plasma carnosine concentration, as determined by the present method, agrees well with the only previously reported value of 5.2 ± 1.8 μM [17]. Carnosine was not detected in normal human plasma by the current method. Previously, carnosine had been detected in the plasma of newborn infants at a level of 3.1 ± 7.5 μM [18], however, it is reported to be absent from normal adult plasma [19–21]. The non-detection of carnosine in adult human plasma, using the present method, supports the supposition that no interfering substances co-elute with the carnosine peak.

Histidine concentrations correlate well with previously reported values for both equine plasma (53.7 ± 3.0 μM) [22] and human plasma (89.12 ± 1.21 μM, 89 ± 12 μM) [23,24]. The

TABLE IV

CONCENTRATIONS OF ENDOGENOUS IMIDAZOLES IN PLASMA

Compound	Concentration (mean ± S.D.) (μM)		
	Equine (n = 8)	Canine (n = 4)	Human (n = 4)
1-Methylhistidine	4.64 ± 0.97	4.64 ± 0.45	5.28 ± 1.47
Histidine	57.30 ± 9.22	59.01 ± 12.00	93.10 ± 11.44
Anserine	N.D.[a]	1.89 ± 0.92	N.D.[a]
Carnosine	11.31 ± 3.90	17.42 ± 9.44	N.D.[a]

[a] N.D. = not detected.

concentration of 1-methylhistidine in equine plasma is reported here for the first time. The level of 1-methylhistidine in human plasma compares favourably with previous data (4 ± 8 μM) [25]. Anserine was not detected in equine or human plasma by this method. No previous publications have reported the detection of anserine in equine plasma. This is presumably indicative of the fact that anserine is only present at very low levels, 2.0 mmol/kg d.m. [26] (*cf.* carnosine), in the skeletal muscle of the horse (the main repository of imidazole dipeptides). Anserine was not detected in normal human plasma by previous investigators [21].

Equine plasma extracts were also injected and analysed at two separate wavelengths both above the 220 nm used for the determination of the plasma imidazoles. At 254 nm and 280 nm no peaks were present in the chromatograms recorded. The lack of any peaks at these two wavelengths is clear evidence for the absence of any interfering compounds such as the purine nucleotides, nucleosides and bases, the amino acids tyrosine, phenylalanine and tryptophan and their metabolites, and indeed any aromatic species.

Although the present method details the determination of only four imidazoles it is also possible to resolve a third histidine-containing dipeptide: homocarnosine (γ-aminobutyryl-L-histidine) which elutes after carnosine at a retention time of 10.4 min. Recovery of homocarnosine from spiked plasma was 99.3 ± 3.4%. Despite a lower limit of detection of 85.5 nM, homocarnosine was not detected in plasma obtained from the three species studied. The absence of homocarnosine from human plasma is in agreement with previous findings from 77 healthy subjects [27]. The authors are not aware of any previous analyses for homocarnosine in equine and canine plasma. The present results are consistent with an earlier study of several species, including man, which showed that homocarnosine is confined to the central nervous system [28]. No detailed assessment of the accuracy and precision of homocarnosine analysis was made owing to its absence from plasma, though the low coefficient of variation for homocarnosine recovery suggests that the analytical reproducibility would be good.

CONCLUSIONS

The HPLC method presented provides a rapid, selective, sensitive and reproducible analysis of carnosine, histidine, anserine and 1-methylhistidine in equine, canine and human plasma. The good compatability between the plasma concentrations of certain imidazoles and the absence of others, here and in previously published data, attests to the validity of this present method.

The present method should be of benefit to investigations of carnosine metabolism and biochemistry in horse, dog and man. In man, several inherited disorders involving histidine dipeptide metabolism are known. Hypercarnosinemia and carnosinuria have been reported in cases of juvenile amaurotic idiocy [29] and have also been associated with progressive neurological disease [21] and urea cycle defects [30]. The current analytical method may prove to be of significant use in the clinical diagnosis off these disorders.

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