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Short communication

High-performance liquid chromatographic determination of N-α-acetyl-L-carnosine in equine plasma

Mark Dunnett

Equine Sports Medicine Unit, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire AL9 7TA, UK

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Abstract

N-α-Acetyl-L-carnosine (NAcCAR) in perchloric acid extracts of equine plasma was assayed by high-performance liquid chromatography on a 3 μm Hypersil ODS (150×4.6 mm I.D.) column eluted with 5 m*M* phosphoric acid–1 m*M* triethylamine, pH 2.58. NAcCAR was isolated by solid-phase extraction on Isolute PRS (propylsulphonyl) columns. The HPLC mean retention time for NAcCAR was 5.9±0.2 min. The recovery from plasma by solid-phase extraction was 93.9–99.7% and lower limit of detection in plasma was 0.18 μ*M*. The normal NAcCAR concentration in equine plasma was 2.4±0.3 μ*M*. The method was applied to the determination of plasma concentrations following oral and intravenous NAcCAR administration.

Keywords: N-α-Acetyl-L-carnosine

1. Introduction

The imidazole dipeptide carnosine (CAR, β-alanyl-L-histidine), and its methylated analogues anserine (β-alanyl-L-1-methylhistidine) and balenine (β-alanyl-L-3-methylhistidine), occur at high concentrations in mammalian skeletal muscle [1], where they primarily act as a H^+ buffers [2,3]. In equine skeletal muscle, which requires a large H^+ buffering capacity, the CAR concentration generally exceeds 100 mmol kg^{-1} dry weight [2,4]. As part of an ongoing investigation of the metabolism and transport of carnosine and its analogues in the horse, one aspect has been to study carnosine pharmacokinetics, and bio-availability from the gastro-intestinal tract following the administration of carnosine by oral and intravenous routes. The importance of the terminal amine group of CAR for transport across the intestinal brush border membrane, and as a binding site during in vivo hydrolysis by carnosinase (EC 3.4.13.3), were studied by administration of the CAR analogue N-α-acetyl-L-carnosine (NAcCAR). However, no high-performance liquid chromatography (HPLC) methods for the determination of plasma NAcCAR could be found in the literature.

Thin-layer chromatography [5] and HPLC with UV detection [6–9] have previously been used to determine NAcCAR and other N-acetyl imidazole compounds in tissues from several species, including the rat, frog and rabbit. These methods however, were not developed for plasma analysis. Furthermore, they lacked the necessary selectivity and sensitivity to quantify low concentrations of NAcCAR in plasma. The analytical method presented combines isocratic reversed-phase HPLC with solid-phase extraction (SPE) to provide rapid, selective

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and sensitive detection of NAcCAR in equine plasma.

2. Experimental

2.1. Materials and instrumentation

Phosphoric acid (H_3PO_4) (85%, w/v), triethylamine (TEA), potassium dihydrogenphosphate (KH_2PO_4) and perchloric acid (PCA) (70% w/v) were obtained from Sigma Chemicals (Poole, UK). NAcCAR was a gift from Dr. E. Hultman, Karolinska Institutet (Stockholm, Sweden). HPLC grade water was obtained from Romil Chemicals (Cambridge, UK). All chemicals were analytical reagent grade unless stated otherwise.

The HPLC system comprised a Hewlett-Packard HP1050 quaternary-gradient pump, a HP1040 diode array detector and Chemstation. Samples were injected via a Rheodyne 7125 injector (200-μl sample loop). A Rheodyne in-line filter (0.5 μm) was inserted between the pump and the injector.

2.2. Sample collection and NAcCAR administration

For normal values, heparinized blood samples were collected from 11 fasting thoroughbred horses (three fillies, eight geldings), aged from 5–9 years. Following a 12-h overnight fast NAcCAR was administered to two horses; by intravenous bolus injection in one (20 mg kg^{-1} body mass), and by naso–gastric intubation (70 mg kg^{-1} body mass) in the other. Blood samples were collected prior to and at intervals after administration.

2.3. Solid-phase extraction

Blood samples were centrifuged for 5 min at 2000 *g* and 4°C. Plasma (250 μl) was deproteinized with 1.2 *M* PCA in 1.0 *M* H_3PO_4 (350 μl), centrifuged and the supernatant collected. The protein residue was re-extracted with 1.0 *M* H_3PO_4 (400 μl) and the supernatants combined. Plasma extracts (250 μl) were loaded onto 1 ml/100 mg Isolute PRS (propylsulphonyl) columns (International Sorbent Technology, Hengoed, UK) previously conditioned with methanol (3×1000 μl) and 1.0 *M* H_3PO_4 (3×1000 μl). Columns were washed with 50 m*M* KH_2PO_4, pH 2.58 (2×250 μl) and air-dried for 2 min. NAcCAR was eluted with 100 m*M* KH_2PO_4, pH 2.58 (2×250 μl). A flow-rate of 0.5 ml min^{-1} was used at all times.

2.4. Chromatography

NAcCAR was eluted from a 3 μm Hypersil ODS (150×4.6 mm I.D.) analytical column protected by a 5 μm Hypersil ODS (20×4.6 mm I.D.) guard column (Jones Chromatography, Hengoed, UK) using a mobile phase comprising 5 m*M* H_3PO_4 and 1 m*M* TEA, pH 2.58 in HPLC grade water. The mobile phase, freshly prepared each day, was filtered to 0.45 μm and degassed by helium sparging prior to and continually during use. The flow-rate was 0.8 ml min^{-1} and the injection volume was 200 μl. Detection was by UV absorbance at 220 nm. Plasma CAR and histidine (HIS) concentrations were determined by ion-pairing HPLC [10].

2.5. Calibration and lower limit of detection

A 1 m*M* stock mixed standard of NAcCAR was prepared by dissolving the required weight of the compound in HPLC grade water (100 ml). Working standards over the concentration range 0.1–100.0 μ*M* were prepared daily by dilution of the stock standard with 100 m*M* KH_2PO_4 buffer, pH 2.58. The lower limit of detection was estimated from regression analysis of the standard curve.

2.6. Recovery and reproducibility

Plasma was spiked at 800 and 0.8 μ*M* with a standard solution of NAcCAR to produce nominal concentrations in the final eluate of 100 and 0.1 μ*M*. Recoveries following solid-phase extraction ($n=5$) at these higher and lower concentrations were determined by comparing measured concentrations in the final eluates with standards at the nominal concentrations. The reproducibility of the SPE–HPLC method was assessed by calculation of the intra- and inter-assay accuracy and precision. Plasma was spiked with NAcCAR at the higher and lower concentrations. Intra-assay precision and accuracy

was derived from the coefficients of variation (C.V.) of replicate analyses ($n=5$) at each concentration within day 1. Inter-assay precision and accuracy was derived from the coefficients of variation (C.V.) of replicate analyses ($n=5$) at each concentration on days 1, 5 and 10.

3. Results and discussion

3.1. Chromatography and SPE

A previous method for the determination of N-acetyl imidazole compounds in tissues [6], and later modified [9], was applied to the measurement NAcCAR in equine plasma. However, application of this method, which utilized a mobile phase consisting of 100 m*M* Na_2HPO_4, pH 2.0 buffer, proved unsuccessful. Spiked plasma samples indicated an interference peak co-eluting with NAcCAR. Furthermore, NAcCAR exhibited poor peak symmetry ($A_s=2.56$). The interference was eliminated and retention times were increased by using a mobile phase comprising 5 m*M* H_3PO_4. Addition of TEA at 20% of the phosphate concentration significantly improved peak symmetry ($A_s=1.07$). However, higher TEA concentrations (>1 m*M*) had an adverse effect. The combination of 5 m*M* H_3PO_4 and 1 m*M* TEA yielded a final pH of 2.58. No further mobile phase pH adjustment was made and the batch-batch reproducibility of pH was excellent. Columns filled with octadecylsilyl (ODS) silica gel material obtained from different sources (e.g., Spherisorb, Apex, Primesphere and Hypersil) were assessed during method development. The latter provided superior resolution and peak symmetry. Separation of a standard solution using the optimized chromatographic conditions resulted in a NAcCAR mean (±S.D.) retention time of 5.9±0.2 min (Fig. 1).

Analysis of PCA plasma extracts, not subjected to SPE, under these chromatographic conditions showed interference with NAcCAR and the presence of large late eluting peaks. For this reason SPE was evaluated for pre-chromatography sample clean-up. The positive charge on the imidazole ring of NAcCAR at acidic pH implies the use of a strong cation-exchanger for SPE. Both benzenesulphonyl and propylsulphonyl bonded phases were assessed,

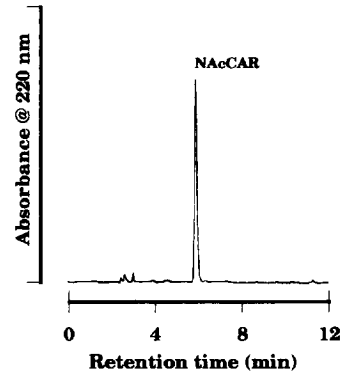

Fig. 1. HPLC separation of a 10 μ*M* NAcCAR standard. Column: 3 μm Hypersil ODS (150×4.6 mm I.D.). Mobile phase: 5 m*M* H_3PO_4, 1 m*M* TEA, pH 2.58. Flow-rate: 0.8 ml min^{-1}. Wavelength: 220 nm. Injection volume: 200 μl.

however, the former exhibited less selectivity, probably due to secondary non-polar interactions with the benzene ring. Obviously the use of the propylsulphonyl phase minimized these potential secondary interactions resulting in a much cleaner sample extract. A comparison of a normal plasma extract before and after SPE is shown in Fig. 2A and 2B. Spiking of a post-SPE plasma extract with authentic NAcCAR produced a single peak co-eluting with that ascribed to endogenous NAcCAR.

3.2. Standards and lower limit of detection

There was a linear relationship between NAcCAR concentration and integrated peak area, as described by the linear regression equation $y=11.713x-0.028$ ($r=0.999$), where y=peak area and x=concentration. The lower limit of detection for NAcCAR in plasma (at a signal-to-noise ratio of 3:1) was estimated as 0.18 μ*M*.

3.3. Recovery and reproducibility

Mean (±C.V.) recoveries of NAcCAR, following SPE, from equine plasma spiked at 800 μ*M* and 0.8 μ*M* were 93.9±5.0% and 99.7±0.5%, respectively. The intra- and inter-assay precision and accuracy of the SPE–HPLC method at the higher and lower concentrations, are given in Table 1. It is possible

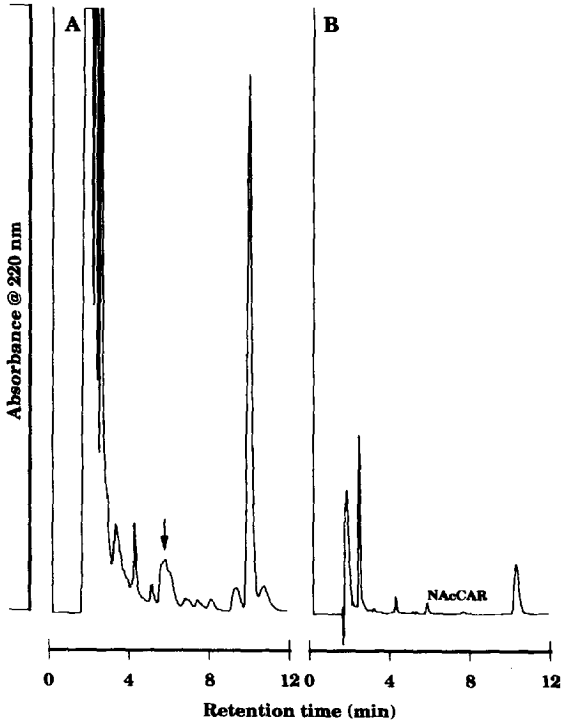

Fig. 2. HPLC separation of a PCA extract of normal equine plasma. (A) Before solid-phase extraction; (B) after solid-phase extraction. HPLC conditions as given in Fig. 1. The position of the obscured NAcCAR peak in the plasma PCA extract prior to solid-phase extraction is indicated by the arrow.

that intra- and inter-assay accuracy and precision, particularly at low concentrations, could be improved through the use of a suitable internal standard. At present however, such a procedure has not been investigated.

3.4. Application to plasma samples

The present method was used to determine normal plasma NAcCAR concentrations in resting horses ($n=11$). The mean ($\pm$S.D.) NAcCAR concentration

Table 1
Intra-assay ($n=5$) and inter-assay ($n=15$) precision and accuracy of the analysis of NAcCAR in equine plasma

Nominal conc. (μM)	Precision (C.V., %)		Accuracy (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
800	3.4	3.6	98.3	93.9
0.8	9.6	11.9	102.4	102.5

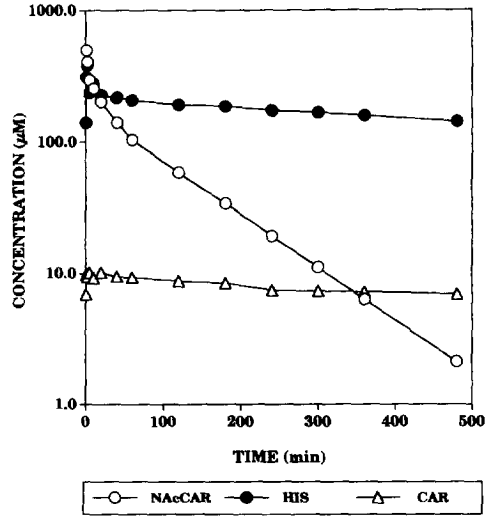

Fig. 3. Plasma concentration–time curves for NAcCAR, HIS and CAR following intravenous NAcCAR administration.

was 2.4 ± 0.3 μM. The authors are unaware of previous determinations of NAcCAR in plasma.

Changes in plasma NAcCAR concentration were determined following intravenous and oral administration. No increase in equine plasma NAcCAR concentration above the normal value was observed following oral administration which suggests that the free terminal amine moiety of carnosine plays an essential role in its uptake from the gastro-intestinal tract. Changes in plasma NAcCAR, HIS and CAR concentrations with time are shown in Fig. 3. These data suggest that the metabolism NAcCAR primarily involves hydrolysis of the peptide linkage, with significantly less deacetylation of the intact peptide.

The combined SPE procedure and isocratic reversed-phase HPLC method described provide a selective, sensitive and reproducible analysis of NAcCAR in equine plasma. The method has been used to establish preliminary data on the bio-availability and metabolism of exogenous NAcCAR, and will be used to further investigate NAcCAR and CAR metabolism in the horse.

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