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Short Communication

Analysis of novel imidazoles from isolated perfused rabbit heart by two high-performance liquid chromatographic methods

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ABSTRACT

The paper reports two analytical high-performance liquid chromatographic methods to detect and quantify cardiac-derived histidyl derivatives. Method A relies on relative hydrophobicities as a basis of separation. Method B is an ion-pairing method in which the compounds are eluted in an entirely different order. Fractions collected from method A have been co-eluted in admixture in method B with authentic reference compounds. Thus the existence of the following imidazole ring-containing compounds derived from heart have been confirmed: N-acetylhistidine, N-acetyl-1-methylhistidine, N-acetylcarnosine, N-acetylhomocarnosine, homocarnosine, anserine, carnosine, balenine. Compounds were found in both tissue samples and perfusates.

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INTRODUCTION

Skeletal muscle contains the well established histidyl dipeptides carnosine (β-alanyl-L-histidine), anserine (β-alanyl-1-methyl-L-histidine) and balanine (β-alanyl-3-methyl-L-histidine). Their relative and total concentrations vary with fibre type and species [1]. We have recently submitted chromatographic and microchemical evidence to suggest that in cardiac muscle a number of N-acetylated derivatives of histidine, both of the free amino acid and of its β-alanyl and γ-aminobutyric acid (GABA) dipeptides, are also present and predominate over their precursors in this tissue. Thus N-acetyl forms of histidine, 1-methylhistidine, carnosine and anserine were indicated [2] as was the N-acetyl form of homocarnosine (γ-aminobutyryl-L-histidine) [3] and lesser quantities of non-acetylated forms of both carnosine and homocarnosine [2,3]. We have also detected related acetylated imidazoles in brain [4] where it is thought they may function as neurotransmitters or neuromodulators in specific regions of the central nervous system (CNS) [5,6].

Wide ranging interest has centred on the possible biological roles of carnosine-related imidazoles principally those relating to pH buffering, antioxidant, membrane-stabilising and enzyme-modulation functions [6]. Our interest in them centres on their apparent ability to calcium-sensitize chemically skinned cardiac muscle [7,8], their α-adrenergic antagonist properties [9] and their synergism with each other in free-radical scavenging [10].

Our approach to the analysis and identification of these compounds has hitherto relied upon the synthesis of putative isomorphs of the compounds concerned, the verification of identities of these by NMR spectroscopy, and the comparison of their chromatographic and chemical properties with those of their isomorphs found in biological extracts. An essential feature of this approach has been the development of selective extraction, chromatographic and detection systems for the compounds in question. Of particular importance has been the use of a chromatographic system that would allow the collection of fractions chemically unchanged, that relied upon the optical properties of the compounds themselves and, crucially for the N-acetyl compounds, does not depend upon either pre- or post-chromatographic N-derivatisation. This was accomplished by the use of reversed-phase octadecylsilane (ODS)-bonded columns eluted isocratically with aqueous phosphate buffer at low pH. Under these conditions the elution order of amino acids and low-molecular-mass peptides, whether or not they are N-acetylated, may be predicted by calculations of their hydrophobic fragmental constants using methods described by Rekker [11]. Previous high-performance liquid chromatographic (HPLC) methods that had relied upon N-derivatisation for the visualisation of muscle imidazoles necessarily failed to detect N-acetyl forms [12–14].

A particular limitation to these studies has been the reliance upon a single chromatographic system for the purposes described. It was therefore necessary to develop a distinct chromatographic system possessed of the attributes described above but which has a different basis of separation for the compounds of interest.

We report here upon the combined use of the original method and an alternative ion-pairing method to confirm the identities of novel cardiac imidazoles both *in situ* in tissues and in perfusates of isolated working heart.

EXPERIMENTAL

Chemicals and biological reagents

Carnosine, anserine, histidine, 1-methylhistidine and N-acetylhistidine were obtained from Sigma (Poole, UK). All other chemicals and biological reagents were purchased either from BDH (Poole, UK) or from Sigma except for those acetylated forms that had to be prepared from their parent compounds. Solvents were of HPLC grade and were obtained from May and Baker (Dagenham, UK). N-Acetylated forms were prepared from their parent bases and identified by NMR spectroscopy and chromatography as described previously [2,4]. Balenine was derived from extracts of whale meat obtained in Japan.

Animals and tissues

Animals (New Zealand White rabbits 2–2.5 kg) were sacrificed by a lethal injection (phenobarbital Sagital 2.5 ml/kg). The heart was rapidly removed, cannulated and perfused with Kreb's buffer (m*M*: NaCl, 119; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 24.8; glucose, 5 or pyruvate, 5; 10 ml/min, 95%O_2–5%CO_2, 35°C) by the method of Langendorff [15]. A pressure transducer on the catheter recorded perfusion pressure, the heart was not paced and tension was recorded using a transducer attached to

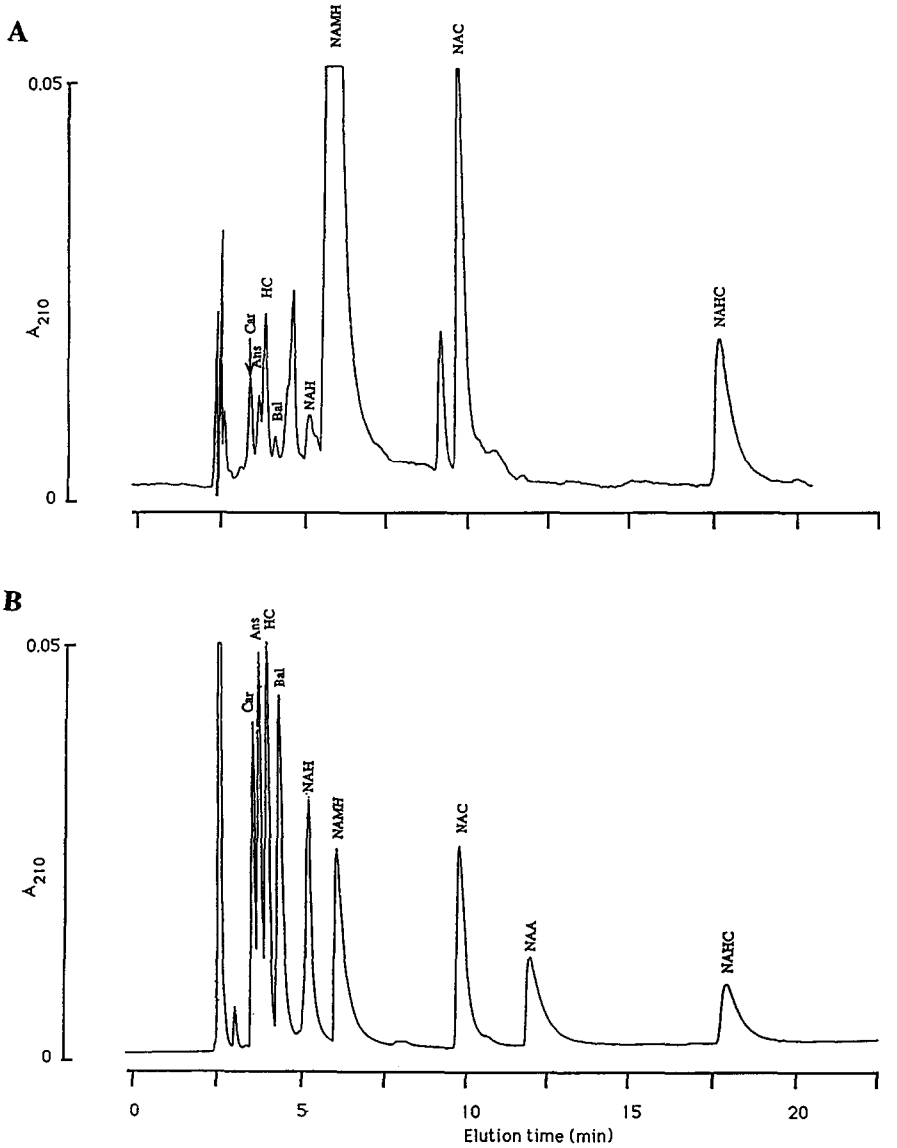

Fig. 1. Chromatograms obtained with method A. Absorbance at 210 nm (ordinate, arbitrary units). (A) Extracted perfusate from Langendorff rabbit heart (see text for details). Peaks are labelled in this, and subsequent figures, as Car (carnosine), Ans (anserine), HC (homocarnosine), Bal (balenine), NAH (N-acetylhistidine), NAMH (N-acetyl-1-methylhistidine), NAC (N-acetylcarnosine), NAHC (N-acetylhomocarnosine). A number of other, as yet unidentified, peaks are present. (B) Authentic reference compounds, labelled as in (A) with NAA (N-acetylanserine) in addition and run as a mixture at concentrations of 0.1–0.15 m*M*; conditions as in (A). Perfusate sample in (A) is $2 \cdot 10^4$ concentrated relative to these standards.

the apex. At appropriate time intervals tissue samples were removed using a pre-cooled, hand-held, powered rotary biopsy instrument (Black and Decker). Portions (10 mg) were flash-frozen in liquid nitrogen prior to extraction. The perfusate was collected and frozen for later examination.

Extraction procedures

Extraction of imidazoles from cardiac muscle tissue was performed using aqueous ethanol exactly as described previously [2]. Extraction of imidazoles from perfusate was performed as follows. Perfusate (up to 1 l) was evaporated to dryness under reduced pressure. The residue was extracted (thrice) using aqueous ethanol (95%, v/v) and the extract was in turn evaporated to dryness under reduced pressure. Material for chromatography was resuspended in distilled water prior to examination for imidazoles by analytical HPLC.

Analytical HPLC

Analytical HPLC was performed using a Gilson/Apple liquid chromatography system (Gilson, Villiers le Bel, France; Apple Computers, Hemel Hempstead, UK). Samples were dissolved in distilled water and injected (20 μl) onto columns (250 mm × 4.6 mm I.D.) packed with Hypersil ODS (5 μm; HPLC Technology, Macclesfield, UK) prior to elution by one of two methods.

Chromatographic method A (reversed-phase analytical HPLC)

Columns were eluted isocratically at room temperature with phosphate buffer (0.1 *M* Na_2HPO_4, pH 2.0), over 20 min at a flow-rate of 1.0 ml/min. Eluates were monitored for UV absorbance at 210 nm. The output was relayed to a Chromatopac C-R1B data processor (Shimadzu, Kyoto, Japan) and areas within peaks were used to provide quantitative data.

Chromatographic method B (reversed-phase–ion-pair analytical HPLC)

Columns were eluted isocratically at 50°C with phosphate buffer (70 m*M* KH_2PO_4, pH 3.3) that contained 10 m*M* 1-heptanesulphonic acid over 60 min at a flow-rate of 1.0 ml/min. Eluates were monitored for UV absorbance at 210 nm and output recorded as described above.

Direct comparison of chromatographic fractions on two HPLC methods

In all cases and for each putative compound standards were run in admixture with their putative isomorphs on each chromatographic system. In addition, fractions corresponding to peaks eluted by method A were collected, evaporated to dryness under reduced pressure and resuspended (water) prior to examination using chromatographic method B. These fractions were run both singly and in admixture with putative standard isomorphs using method B. Fractions collected after elution using method B could not likewise be run using method A since the presence of 1-heptanesulphonic acid interferes with that chromatographic method.

RESULTS

The two different chromatographic systems provided two quite different patterns of elution of the compounds in question. Thus whereas in method A (Fig. 1B) the order of elution was Car, Ans, HC, Bal (=ophidine), NAH, NAMH, NAC, NAA, NAHC, in method B (Fig. 2B) the elution order was NAMH, NAH, NAA, NAC, NAHC; Ans, Car, Bal, HC[a]. These results are the consequence of the different bases of separation between the two chromatographic systems (see Discussion). In each case and with both methods authentic reference compounds were found to run in admixture with their putative biological isomorphs (data not shown). Moreover, fractions collected from eluates of extracts of biological samples using method A when run, both singly and in admixture, with authentic reference com-

[a] *Abbreviations*: carnosine (Car), anserine (Ans), homocarnosine (HC), balenine (=ophidine) (Bal), N-acetylhistidine (NAH), N-acetyl-1-methylhistidine (NAMH), N-acetylcarnosine (NAC), N-acetylanserine (NAA), N-acetylhomocarnosine (NAHC).

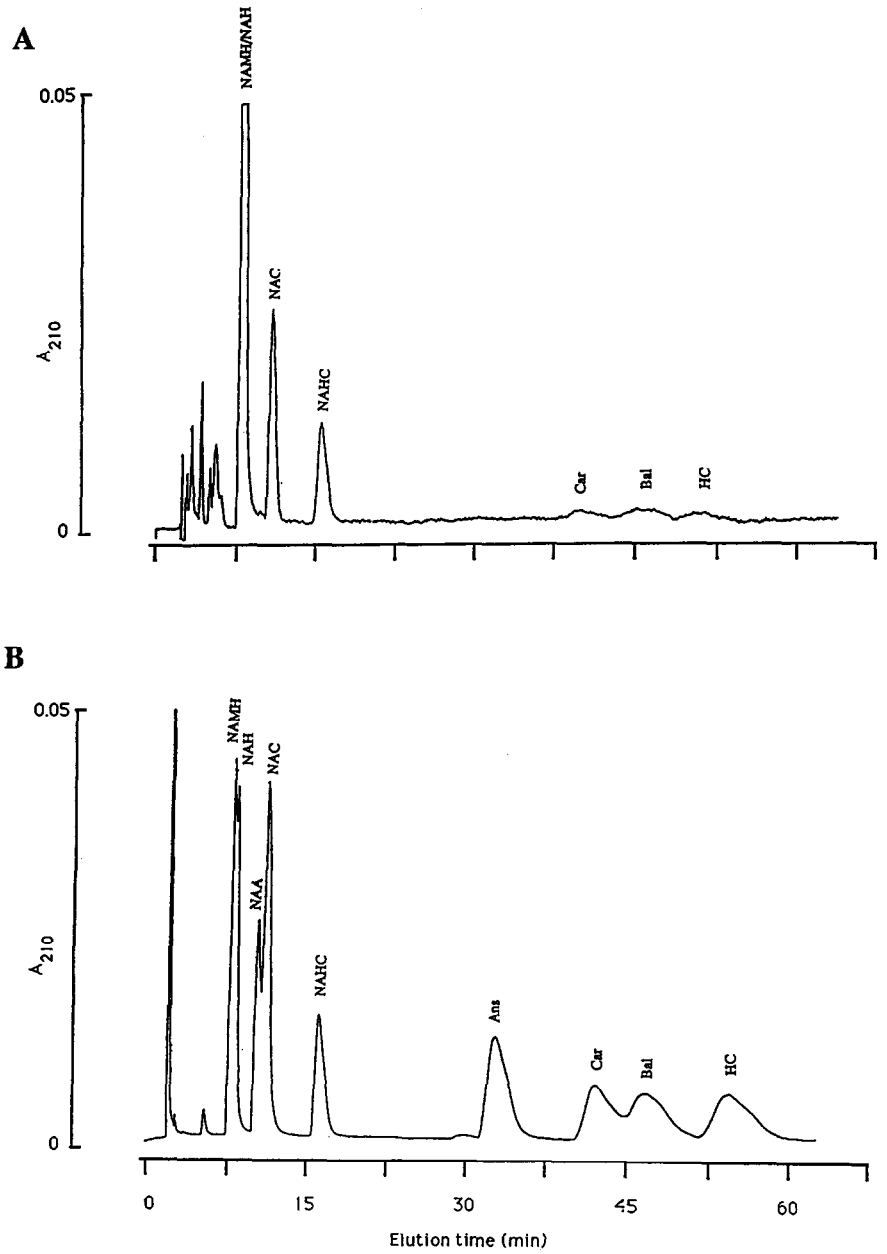

Fig. 2. Chromatograms obtained with method B. Absorbance at 210 nm (ordinate, arbitrary units). (A) Extracted perfusate, as in Fig. 1. Peaks labelled as in Fig. 1 except that NAMH/NAH cannot be resolved satisfactorily with this method. (Collection of this fraction re-run on method A confirms the presence of both, but predominantly NAMH.) (B) Authentic reference compounds, labelled as in (A), were run as a mixture as described in Fig. 1.

pounds corresponded to these reference compounds and eluted with them as a single peak when run in admixture. Thus we are able to confirm, on the basis of common chromatographic properties on two distinct chromatographic systems, the existence of the following cardiogenic imidazoles: NAH, NAMH, NAC, NAHC, Car, HC, Bal. Neither Ans nor NAA were found in these samples.

These compounds were found in various pro-

portions both in extracts of cardiac muscle tissue (not illustrated; see also refs. 2 and 3) and in perfusates of isolated working heart (Figs. 1A and 2A). No peaks corresponding to compounds of interest, or interfering with their peaks, were found in extracts of buffer that had not perfused the heart. The two chromatographic methods gave similar quantitative results for the perfusate sample. For the example illustrated these were as follows (for methods A and B, respectively): NAMH, 550 and 475 n*M* (method B measures NAMH and NAH combined); NAC, 28 and 25 n*M*; NAHC, 20 and 24 n*M*; Car, 11 and 40 n*M*; HC, 11 and 40 n*M*. (The variability of repeat measurements of peak areas for a single sample is less than 1% for both methods.) The discrepancies for Car and HC may reflect their incomplete resolution in method A. These concentrations, as could be expected, are well below those detected in cardiac tissue (see refs. 2 and 3) where the compounds are found in the range 0.1–10 m*M*. The physiological factors, such as flow-rate and others, which will determine the exit rate of these compounds from the heart into the perfusate, are beyond the scope of this paper.

DISCUSSION

Two distinct HPLC methods have been used to analyse cardiac muscle-derived imidazole substances, some of them acetylated and novel. The first (method A, reversed-phase HPLC) relies on the elution from a non-polar stationary phase (octadecyl silica) with a polar mobile phase (aqueous phosphate at low pH) of a number of closely related histidyl compounds that vary slightly in a number of respects. These include aliphatic chain length, methylation (or not) at one of two positions in the histidyl imidazole ring and whether acetylated (or not) on the N-terminal amino nitrogen atom. Under these chromatographic conditions, it has been demonstrated that for homologous amino acids and low-molecular-mass peptides there is a linear relationship between the capacity factor (log k') of the column and the hydrophobic fragmental constant of the eluted compound [11,16]. Thus method A separates on the basis of hydrophobicity.

In contrast, method B (reversed-phase–ion-pair HPLC) relies upon the interaction of a hydrophobic surfactant anion (1-heptanesulphonic acid) with a solute cation (the pH-dependent protonated terminal amino group) to form a "solvophobic" uncharged group which then absorbs onto the non-polar stationary phase [17,18]. This "ion-pair" may be eluted from the column in a temperature-dependent manner using an eluate of appropriate ionic strength [19]. Clearly, N-acetyl derivatives are incapable of ion-pair formation and their retention behaviour is independent of the concentration of hydrophobic surfactant anion.

Two independent HPLC methods have been used to examine cardiogenic imidazole compounds. A direct comparison of the behaviour of authentic reference compounds and their putative biological equivalents has been made. Direct comparison of a number of fractions corresponding to these has been accomplished. Biological extracts have shown identical properties to a number of reference compounds on each chromatographic system. The elution in admixture of fractions collected from one system with appropriate reference compounds in another system provides persuasive evidence of the identities of compounds in those fractions.

The full biological role of the muscle and brain imidazoles is the subject of extensive speculation but nevertheless remains obscure [1,5,6]. Data from this laboratory suggest that they may have a role in calcium-sensitizing the contractile apparatus of cardiac muscle [7,8] and that they may modulate adrenergic receptor function in blood vessels [9]. We have recently presented evidence that histidine-containing compounds display potent synergistic antioxidant activity at near physiological concentration [10]. It is therefore possible that a combination of some or any of the above effects may have important implications for cardiac function both in health and disease. The use of the combined chromatographic methods described above may prove useful in the study of such functions.

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