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Note

Rapid and sensitive method for the determination of arterial–venous differences and leg efflux of 3-methylhistidine using ion-pair high-performance liquid chromatography and post-column fluorescence derivatization

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Methylation of histidine occurs after its incorporation into the peptide chains of actin and myosin [1–3]. After degradation of these proteins, the 3-methylhistidine (3-MH) liberated is not recycled but quantitatively excreted in the urine [4]. 3-MH has thus been proposed as an index of skeletal muscle protein catabolism [5]. Recently, this hypothesis has been re-evaluated and the quantitative importance of non-skeletal muscle contribution to 3-MH excretion has been emphasized [6,7]. In fact, the increased urinary output of 3-MH in catabolic conditions does probably reflect, to a major extent, visceral protein degradation and non-skeletal muscle breakdown [8]. Since the concentration of 3-MH in plasma is low, a method based on the ninhydrin reaction is probably not sensitive enough. An improved selectivity may, however, be achieved by pre-incubation

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with formaldehyde [9], since the latter compound markedly reduces the high and otherwise interfering histidine peak in the chromatogram.

Using ion-pair high-performance liquid chromatography (HPLC) with post-column *o*-phthalaldehyde (OPA) derivatization and fluorescence detection, a fast, sensitive and simple alternative was introduced by Friedman et al. [10]. A further increase in sensitivity may be obtained by using a fluorescamine reagent [11]. An optimal sensitivity is crucial for measuring the small arterial–venous (A–V) differences for 3-MH in plasma [8,9], used for estimation of efflux for 3-MH from a part of the body [6,8].

In the present paper we describe a modification of the method by Friedman et al. [10], since the original method in our hands did not yield an optimal separation for 3-MH from interfering amino acids. Data on A–V differences and efflux from the leg for normal healthy men are given and discussed in relation to earlier data from the literature [8,9,12].

EXPERIMENTAL

Apparatus

The apparatus consisted of a ConstaMetric III solvent delivery pump from LDC (Riviera Beach, FL, U.S.A.), a Rheodyne Model 7120 injection valve (Berkeley, CA, U.S.A.) with a 100-μl loop and a 250×4.6 mm steel column from Supelco (Bellefonte, PA, U.S.A.) packed with 7-μm Nucleosil C_{18} (Macherey Nagel, Düren, F.R.G.). A quard colum of 260×4.6 mm packed with Polygosil 60 C_{12} (particle size 25–40 μm) was used. An Altex Model 100A pump (Berkeley, CA, U.S.A.) was equipped with an internal pulse damperer for the post-derivatization reagent. The fluorescence was measured with a Fluoro-Monitor III from LDC using an excitation wavelength of 350 nm and a 420-nm emission filter. A Linear Model 255 potentiometric recorder was used to register the fluorescence.

Materials

The chemicals used were of analytical-reagent grade and all solvents were prepared with ultrapure water (Milli-Q water purification system, Millipore, Bedford, MA, U.S.A.). Boric acid, potassium hydroxide, methanol, acetic acid, trichloroacetic acid (TCA) and 30% Brij 35 were purchased from Merck (Darmstadt, F.R.G.). 2-Mercaptoethanol, OPA and 3-methyl-L-histidine were obtained from Sigma (St. Louis, MO, U.S.A.). Ethanol (AB Swedish Spirit) and 1-heptanesulphonic acid sodium salt were from Fisons (Loughborough, U.K.).

Samples

The seven subjects participating in the study were healthy normal-weight men, aged 20–37 years, from the city fire brigade, without a previous history of serious illness or medical treatment. Normal serum creatinine levels were found and no previous history of renal disease was known. All samples were taken in the morning after 12 h fasting. Prior to the study all subjects were fed ad libitum, since any meat present in the diet would only affect the absolute plasma level of 3-MH and not A–V differences or flux data.

Plasma samples were obtained from whole blood (heparinized) by centrifugation at 1500 *g* for 10 min. A 1.0-ml plasma sample was then immediately deproteinized with 0.5 ml of 10% TCA. After vortex-mixing and standing at 4°C for 1 h, the sample was centrifuged at 1500 *g* for 10 min. The clear supernatant was then diluted with the mobile phase as for standards before injection. The protein-free samples were stored at −20°C until analysis, although no loss of 3-MH was seen if the sample was kept at 25°C for two weeks. For blood flow measurements thin catheters were introduced percutaneously under local anaesthesia (Xylocain®, 10 mg/ml) into the femoral artery and vein on both sides. Leg blood flow was determined using a dye dilution technique based on constant infusion of indocyaniue-green (cardio-green) in the femoral artery according to Jorfeldt and Wahren [13]. Plasma flow was estimated from blood flow values by correction for the haematocrit value of the blood sample.

The use of leg plasma flow, instead of whole blood flow, was preferred since flux data over the erythrocyte membrane for 3-MH are not yet available and the erythrocyte level may represent a more stable 3-MH portion than that of plasma.

To evaluate the distribution of 3-MH between erythrocytes and plasma, erthrocytes were washed twice with cold 0.9% sodium chloride. Erythrocytes were then haemolized by incubation with 2 vols. of ultrapure water for 5 min. The haemolyzate was then treated as for plasma.

Methods

Preparation of the OPA reagent was achieved according to Friedman et al. [10]. They recommended that the reagent should be prepared daily. We experienced, however, if a freshly prepared reagent was used, that an unstable baseline was seen. This effect could be eliminated if the OPA reagent was allowed to settle for at least 12 h before use. No major loss in sensitivity was thereafter seen over a period of five days. The flow-rate for the OPA reagent was 1.0 ml/min. The mobile phase was prepared as follows: to a solution of 1.0 l of 5 m*M* sodium heptanesulphonate, 4.0 ml concentrated acetic acid were added and the pH was adjusted to 4.0 with concentrated sodium hydroxide. The solution was then filtered through a 0.45-μm membrane filter and methanol was added to a concentration of 4% (v/v). The flow-rate was 0.8 ml/min.

A standard solution of 3-MH was prepared in ultrapure water and stored at −20°C. Standard samples were diluted 1:1 with the mobile phase before analysis.

RESULTS AND DISCUSSION

The constituents of the mobile phase are prime regulators of separation capacity of an HPLC system. By increasing the pH, adding methanol or reducing the carbon chain of the alkylsulphonate molecule, reduction in retention time of 3-MH may be achieved. The presence of acetic acid also shortens the retention time. An acceptable separation for 3-MH was achieved by increasing the pH from 3.2 to 4.0, as originally described [10]. The retention times of 3-MH and tryptophan (the last peak in the chromatogram) obtained were, however, 30 and 140 min, respectively, and considered too long for practical purposes. Suitable reten-

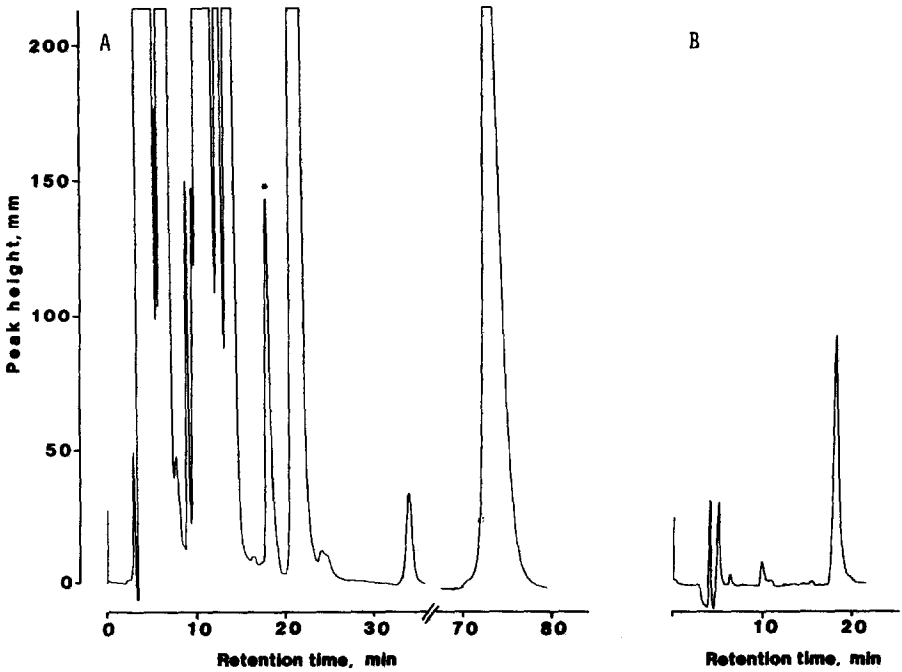

Fig. 1. Chromatograms of (A) an unspiked plasma sample and (B) a standard solution of 3-methylhistidine (*).

tion times were, however, achieved by addition of methanol to a final concentration of 4% (see *Methods*), whereas a further increase in methanol concentration produced a too compact chromatogram with an inadequate separation for 3-MH.

The precision of the method was estimated from duplicate determinations of ten plasma samples. The range for the 3-MH concentration was 2.10–5.58 μmol/l. From a mean of 3.33 μmol/l and S.D. of 0.10 μmol/l (calculated from duplicate analysis) a coefficient of variation of 2.95% was obtained. Recovery of pure 3-MH added to plasma samples was $96 \pm 2\%$ (S.D.). The sensitivity (signal-to-noise ratio of 2) was 3.2 pmol, calculated from a standard sample of 100 pmol. The fluorescence of 3-MH was linear throughout the range 25 pmol to 10 nmol, corresponding to a plasma concentration of 0.5–200 μM ($y = 0.28x - 1.58$). Chromatograms of a typical unspiked plasma sample and a standard solution of 3-MH are shown in Fig. 1. As can be seen from the figure an excellent separation of 3-MH from other amino acids is obtained. The only drawback of the present method is a late-eluting peak with a retention time of 70 min corresponding to synthetic tryptophan. This problem could, however, be solved by pre-incubating the plasma sample with tryptophanase (data not shown). Using the present procedure sample injections can easily be timed to avoid interference of tryptophan with 3-MH, without any major effect on efficiency.

In analysis of efflux data for 3-MH from a leg it is assumed that the compound is equally distributed between plasma and erythrocytes [8;12]. The accuracy of this statement is of vital importance since earlier efflux data were based on plasma

TABLE I

NORMAL ARTERIAL AND VENOUS CONCENTRATIONS, A-V DIFFERENCES AND EFFLUX FROM THE LEG FOR 3-MH IN SEVEN HEALTHY MALE SUBJECTS

Subject	Concentration (μmol/l)			Plasma flow (l/min)	Net efflux (nmol/min)	Net efflux (nmol/min per m^2 LSA★
	Arterial	Venous	A–V			
1	4.77	5.11	−0.34	0.15	51.0	151
2	3.20	3.55	−0.35	0.25	87.5	248
3	4.34	4.79	−0.45	0.16	72.0	205
4	4.84	5.18	−0.34	0.19	64.6	165
5	3.44	3.55	−0.11	0.27	29.7	91
6	3.76	3.97	−0.19	0.18	34.2	103
7	4.25	4.70	−0.45	0.22	99.0	277
Mean	4.08	4.40	−0.32	0.20	63.0	177
S.E.M.	0.24	0.27	0.047	0.02	9.8	26.5

★LSA = leg surface area, calculated as 18% of total body surface area.

3-MH determinations and whole blood flow measurements. The concentration of 3-MH in erythrocytes measured in five of our subjects was 95–98% of that for plasma of the same sample. Extrapolation of data from plasma 3-MH determinations and whole blood flow measurements for the leg to efflux data for 3-MH thus seems acceptable. As stated in *Materials* plasma flow was, however, preferred by us. The total body production of 3-MH from muscle tissue, calculated from one efflux leg muscle tissue data, are also quantitatively reasonably appropriate as compared to the amounts excreted of 3-MH in the urine for the same subject (data not shown). Data on A–V differences and efflux for 3-MH from the leg are given in Table I. If compared to earlier reports, the difference in expressing efflux data (nmol/min) and nmol/min per 100 g tissue [8,12] is related to the invasive technique of measuring blood flow of the present investigation. In the previous reports leg blood flow was estimated by pletysmography [8,12].

The arterial and venous concentrations and A–V differences for 3-MH in our healthy subjects were in agreement with those in a previous report [9], although higher and more variable values have also been published [8,12]. The differences could, in part, be related to the technique for blood flow measurements (as earlier discussed) and selection of reference material, which in the previous studies consisted of elderly subjects undergoing minor elective surgery [8,9,12]. Furthermore, the excellent selectivity and sensitivity of our method probably contributed to the reduced inter-individual variation (standard error of the mean, S.E.M.) of the different 3-MH variables investigated (Table I). The present method thus offers a simple and precise quantitation of plasma 3-MH at the pmol level and thereby a fair judgement of muscle tissue breakdown in various conditions.

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