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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 3-METHYLHISTIDINE IN HUMAN URINE

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SUMMARY

An internally standardized method for the determination of 3-methylhistidine in human urine is presented. This methylated amino acid and the chemically analogous internal standard 3-ethylhistidine were isolated from human urine specimens using small columns of cation-exchange resin. Quantification was accomplished by high-performance liquid chromatography using post-column derivatization with *o*-phthalicdicarboxaldehyde–2-mercaptoethanol followed by fluorometric detection. Sample-to-sample and day-to-day reproducibility were shown to have respective relative standard deviations of 2 and 5% for a human urine specimen containing 250 nmol/ml 3-methylhistidine when using 250 μl urine per analysis. The chromatographic separation was evaluated in terms of various peak descriptors (capacity factor and retention time) and "Chromatographic Figures of Merit" (peak symmetry and chromatographic efficiency). The utility of the method was demonstrated by its successful application to 1000 human urine specimens.

INTRODUCTION

The methylated amino acid 3-methylhistidine is a component amino acid of skeletal muscle actin and myosin [1]. It has been proposed that muscle protein degradation can be correlated with 3-methylhistidine levels in urine [2,3]. Despite data disputing the validity of this approach [4,5], the appeal of a non-invasive means to evaluate protein turnover has spawned interest in development of analytical methods for determining urinary 3-methylhistidine.

Several analytical approaches have been pursued for the quantification of 3-methylhistidine in biological tissues and fluids including colorimetry [6], amino acid analysis [7–12], thin-layer chromatography (TLC) [13], gas chromatography (GC) [14–16], gas chromatography–mass spectrometry (GC–MS)

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[17,18], and high-performance liquid chromatography (HPLC) [19–23]. Careful inspection of these methods reveals the following: the ease and low cost of colorimetry and TLC must be balanced against their inherent lack of sensitivity and specificity; GC of amino acids requires pre-injection derivatization; GC–MS hardware may not be available. Those methods based on amino acid analysis identify 3-methylhistidine as part of an amino acid profile, making this approach very time-consuming. HPLC is an attractive option for reasons of speed and economy, but published methods attempt to quantify a small, chromatographically unresolved peak representing 3-methylhistidine from among many amino acid residues present in much greater amounts in the sample matrix. The separation is frequently accomplished using solvent gradients. Among all of these methods, experimental internal standardization is infrequent.

We recently reported a rapid, isocratic, internally standardized HPLC method for the quantification in plasma and urine of another methylated amino acid, 6-N,N,N-trimethyllysine [24]. Based on this methodology, we have since developed a rapid, isocratic, internally standardized HPLC method to determine 3-methylhistidine in human urine. This was accomplished by (1) synthesizing an appropriate internal standard (3-ethylhistidine), (2) modifying our sample simplification procedure, and (3) using a more efficient HPLC column. We have successfully applied this new urinary 3-methylhistidine method to 1000 human urine specimens.

To monitor the quality of the chromatographic procedure throughout the duration of the study, several peak descriptors and "Chromatographic Figures of Merit" (CFOM) were routinely calculated for the 3-methylhistidine and 3-ethylhistidine peaks in the analyses performed. The traditional peak descriptors calculated include capacity factor (k') and peak height. The graphic method of Foley and Dorsey [25] for characterization of exponentially modified Gaussian signals [26,27] permitted convenient calculation of CFOM for large numbers of experimental samples. These calculations were used to quantify the stability and efficiency of the chromatographic procedure.

EXPERIMENTAL

Equipment and materials

The chromatography hardware, data handling equipment, and supplies were identical with those previously described [24]. The chromatographic separation was accomplished on a Radial-Pak C_{18} analytical column (10×0.8 cm, 5 μm particle size) contained in a radial compression module (RCM-100) both purchased from Waters Assoc. (Milford, MA, U.S.A.). ^{1}H NMR spectra were recorded with a Bruker (Billerica, MA, U.S.A.) WH-180/270 pulsed Fourier transform NMR spectrometer. Pyridine (HPLC grade) was purchased from Aldrich (Milwaukee, WI, U.S.A.).

The HPLC eluent and derivatization reagent solutions were identical to those used for 6-N,N,N-trimethyllysine determinations [24].

3-Methylhistidine, 1-methylhistidine, histidine, N^2-acetylhistidine, lysine, carnosine (β-alanylhistidine), and anserine (β-alanyl-3-methylhistidine) were

purchased from Sigma (St. Louis, MO, U.S.A.). 6-N-Methyllysine and 6-N,N-dimethyllysine were purchased from Cyclo Chemical (Los Angeles, CA, U.S.A.). 6-N,N,N-Trimethyllysine was synthesized as described [28]. N^G-Methylarginine, N^G,N^G-dimethylarginine, and N^G,N'^G-dimethylarginine were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). Arginine, phenylalanine, and ornithine were purchased from Nutritional Biochemicals (Cleveland, OH, U.S.A.). Iodoethane was purchased from Aldrich. Dowex 50-X8 (200–400 mesh, H^+ form) was purchased from Sigma and converted into the pyridinium form with 25% (v/v) pyridine in reagent-grade water.

Preparation of 3-ethylhistidine internal standard

To a glass pressure vessel equipped with a magnetic stirring bar were added 30 ml of methanol, 5.0 g ($2.3 \cdot 10^{-2}$ mol) of N^2-acetylhistidine, and 4.1 g ($1.3 \cdot 10^{-2}$ mol) of $Ba(OH)_2 \cdot 8H_2O$. This mixture was stirred for 5 min, then treated with 2 ml ($2.5 \cdot 10^{-2}$ mol) of iodoethane. The bottle was tightly sealed and its content was warmed at 85°C with continuous magnetic stirring for 48 h. The flask was cooled to room temperature and 0.8 ml ($1.4 \cdot 10^{-2}$ mol) of concentrated sulfuric acid were added. The barium sulfate precipitate was separated from the reaction solution by centrifugation at 1600 *g*. The supernatant liquid was decanted and evaporated under vacuum, leaving a yellow-brown syrup. This was dissolved in 50 ml of 6 *M* hydrochloric acid and the solution boiled under reflux for 12 h. This hydrolysis solution then was cooled to room temperature, evaporated to a brown syrup under vacuum, and subsequently diluted with 10 ml of water.

The solution was applied to a 2.0×45 cm column of Dowex 50-X8, 200–400 mesh (pyridinium form) cation-exchange resin in equilibrium with 0.25 *M* pyridine (based on an assumed concentration of 12 *M* for neat liquid pyridine). The column was washed with 200 ml of 0.25 *M* pyridine at a flow-rate of 2 ml/min; 10-ml fractions were collected. The column then was eluted with 900 ml of 2.5 *M* pyridine.

Fractions were examined for ninhydrin reactivity by a modification of a published method [29] in which sodium cyanide was omitted from the sodium acetate buffer. Weakly ninhydrin-reactive fractions were found in tubes 21–24 and 34–39; a very strongly ninhydrin-reactive peak was found in fractions 84–103. This last series of tubes were combined and evaporated under vacuum to leave a transparent, pale green glass which crystallized on standing overnight in a freezer at −20°C. The crystals were suspended in dry acetone and collected by vacuum filtration; yield: 1.27 g ($6.9 \cdot 10^{-3}$ mol, 30% based on a calculated formula weight for $C_8H_{13}N_3O_2$ of 183). The identity of the product as 3-ethylhistidine was confirmed by ^1H NMR spectrometry by analogous comparison of synthesis product spectra with spectra of commercially available 3-methyl- and 1-methylhistidine. ^1H NMR [^2H_2O solution, acetone included as internal reference (defined as 2.222 ppm)]: 2.050–2.128 ppm (t, 3H, $—CH_2CH_3$), 3.777–3.891 ppm [m, 2H, $—CH_2CH(N^2H_2)—$], 4.625–4.732 ppm [m, 1H+2H, $—CH_2CH(N^2H_2)—$ and $—CH_2CH_3$], 7.745 ppm [s, 1H,=N—CH=C(CH_2—)—N(C_2H_5)—], 8.347 ppm (s, 1H, $—N^2H—CH=N—$). The isolated material was chromatographed as described below and found to elute as a single *o*-phthalicdicarboxaldehyde —2-

mercaptoethanol-reactive peak containing more than 99% of the total integrated peak area. The chromatogram showed no baseline disturbance in the vicinity of the expected 3-methylhistidine peak.

Sample preparation

Human urine or 3-methylhistidine standard solution (30—300 nmol/ml, 250 μl) and 250 μl 3-ethylhistidine internal standard solution (200 nmol/ml) were combined. The tube was vortexed and the contents applied to a 3×0.5 cm column of Dowex 50-X8 resin (200–400 mesh, pyridinium form, water-washed) contained by a pasteur pipet. The column was washed with 2 ml of pyridine–water (2:98, v/v) and then eluted with 4.5 ml of pyridine–water (20:80, v/v). The final 4.5 ml of effluent were collected in 13×100 mm glass test tubes and the tube contents were evaporated to dryness. The resulting residue was reconstituted in 300 μl of 0.085% (v/v) phosphoric acid, vortexed for 5 s, and 3 μl were injected into the chromatographic system.

Quantification

Standard curves of 3-methylhistidine/3-ethylhistidine peak-height ratios versus concentration were established daily. The 3-methylhistidine concentration in experimental samples was interpolated from a least-squares regression line through standard data points. All standards and experimental samples were analyzed in duplicate.

Recovery and reproducibility studies

The recovery of 3-methylhistidine, 3-ethylhistidine, and various other amino acids and dipeptides from the cation-exchange resin column was examined by external standardization. Each analysis was performed in replicate using either 3-methylhistidine or 3-ethylhistidine as external standards.

Studies were undertaken to demonstrate the reproducibility of the entire urinary 3-methylhistidine determination procedure. Two normal human urine samples were analyzed in replicates of six on three separate days to determine the sample-to-sample and day-to-day reproducibility. A representative human urine sample carried through the analytical procedure was injected into the HPLC system ten times to establish injection-to-injection reproducibility.

RESULTS AND DISCUSSION

This method for the determination of 3-methylhistidine in human urine has two important features: (1) it is rapid and (2) it is internally standardized.

The chromatographic separation time per sample for the described HPLC method is between 8 and 13 min, depending on the use history of the analytical column. As a comparison, an alternative method based on ion-exchange amino acid analysis [7] has a chromatographic separation time of 3.5 h per sample. The HPLC methods examined [19–23] require separation times of 15–40 min. To illustrate the overall speed and stability of our method, a single operator analyzed (in duplicate) 700 human urine specimens within a 3.5-week period of continu-

ous HPLC sampling. This included all sample preparation, chromatography and calculations.

Internal standardization is commonly ignored despite advantages in accuracy, precision, ease of monitoring reproducibility, and the ability to correct for small variability in sample handling [30]. We recognized that accurate quantification of 3-methylhistidine would require a chemically appropriate internal standard. 3-Ethylhistidine was expected to exhibit both usefully greater retention than 3-methylhistidine under conditions of reversed-phase ion-pair HPLC and functional group acidities similar to those of 3-methylhistidine during all phases of sample isolation. A modification of a published regio-selective synthesis of 3-methylhistidine [31] produced a product mixture containing troublesome quantities of the 1-ethyl analogue. Subsequent preparations of 3-ethylhistidine were conducted via the simpler two-step procedure described above. Imidazole-N-alkylation under these conditions was found to be highly regioselective, favoring reaction at the 3-position. We made no empirical investigation of reaction conditions which affected this regioselectivity. As results varied from 80% excess 3-N-alkylated versus 1-N-alkylated product to virtually 100% 3-N-alkylation, semipreparative resolution of these two compounds may be required. This can be accomplished on columns of Dowex 50-X8 (200–400 mesh, pyridinium form) cation-exchange resin loaded to 10% of exchange capacity and eluted with a linear pyridine concentration gradient formed by mixing five column volumes each of 0.25 *M* and 2.5 *M* pyridine. Employing an analogue (3-ethylhistidine) of 3-methylhistidine as the internal standard permitted simultaneous removal of the majority of the extraneous amino acids and quantitative recovery of 3-methylhistidine and internal standard from the small ion-exchange columns. None of the HPLC methods cited [19–23] is internally standardized.

The use of Dowex 50 pyridinium form resin to remove neutral and acidic amino acids from a biological sample matrix has been previously described [8,15]. This resin and a pyridine step gradient provided fractionation of the mixture of amino acids found in urine permitting selective elution of 3-methylhistidine and 3-ethylhistidine. Table I shows the chromatographic retention times and recovery of representative amino acids/dipeptides using the described procedures. Under these separation conditions, the amino acid lysine is the only endogenous chromatographically interfering amino acid. As Table I shows, the Dowex resin removes lysine, while the recoveries of 3-methylhistidine and 3-ethylhistidine internal standard are quantitative. Histidine recovery is variable; however, further elution with 20% (v/v) pyridine would permit its quantitative recovery if desired.

Various studies were undertaken to document the precision of this method. Two normal human urine samples each prepared in replicates of six on three separate days and carried through the analytical procedure yielded the following results: the sample-to-sample variability study values (day one) were 258 ± 4 and 177 ± 3 nmol/ml (mean $\pm$ S.D., $n = 6$) for relative standard deviations of 2%. The day-to-day variability study of these same urine specimens over the three days gave values of 251 ± 12 and 175 ± 3 nmol/ml (mean $\pm$ S.D., $n = 18$) for respective relative standard deviations of 5 and 2%. Ten replicate injections of a represen-

TABLE I

RECOVERY OF SELECTED AMINO ACIDS AND DIPEPTIDES FROM THE CATION-EXCHANGE RESIN SYSTEM

Amino acid/dipeptide	n	Retention time (min)	Recovery (mean ± S.D.) (%)
Phenylalanine	2	3.9	0
Ornithine	2	6.1	0
Histidine	6	5.9	70 ± 30
1-Methylhistidine	6	5.9	94 ± 6
3-Methylhistidine	6	6.8	101 ± 2
3-Ethylhistidine	6	11.2	103 ± 3
Lysine	2	7.4	0
6-N-Methyllysine	2	9.2	0
6-N,N-Dimethyllysine	2	9.7	0
6-N,N,N-Trimethyllysine	2	9.9	0
Arginine	2	16.5	0
N^G-Methylarginine	2	18.7	0
N^G,N^G-Dimethylarginine	2	20.5	0
N^G,N'^G-Dimethylarginine	2	19.0	0
Anserine	2	16.5	0
Carnosine	2	18.5	0

tative urine gave a value of 182 ± 2 nmol/ml (mean ± S.D.) for a relative standard deviation of 1%.

During this study, as with 6-N,N,N-trimethyllysine [24], we needed a basis for evaluation of both the quality of the chromatographic separation of the compounds of interest and the performance of analytical columns throughout their useful lifetimes. Several methods of peak and column performance characterization have been reviewed recently [32]. These authors confirmed earlier findings [33] that peak characterization calculation procedures derived from assumptions of purely Gaussian peak shape may significantly overestimate the separation efficiency of the tailing peaks often encountered during chromatographic experiments. The exponentially modified Gaussian (EMG) model proposed by Gladney et al. [34] and subsequently examined by others [35–37] is now well accepted. This model holds that a chromatographic peak is well represented as the convolution of a Gaussian probability distribution with an exponential decay process.

Recently, Foley and Dorsey [25] described and validated a new method for calculation of a series of CFOM. They adduced a series of least-squares fitted equations derived from the EMG peak model. Their approach is easily understood and easily programmed. Only the peak retention time (t_R) and the times on both the leading and trailing edges of peaks at which the signal amplitude reaches 10% of that at t_R must be determined. We tested this calculation method and found it to behave well when applied to actual chromatographic data.

The peak descriptors and CFOM of EMG peaks most useful to chromatographers include k' (capacity factor), σ (standard deviation of the parent Gaussian function), τ (time constant of the exponential modifier), the ratio τ/σ (a fun-

TABLE II

PEAK DESCRIPTORS AND CHROMATOGRAPHIC FIGURES OF MERIT (CFOM) FOR 3-METHYLHISTDINE AND 3-ETHYLHISTIDINE AS DETERMINED IN A SINGLE CHROMATOGRAPHIC RUN CONTAINING A FIVE-POINT STANDARD CURVE AND 62 HUMAN URINE SPECIMENS, ALL PERFORMED IN DUPLICATE

	3-Methylhistidine	3-Ethylhistidine
Number of injections	134	134
Number of injections suitable for analysis	132	134
k'	16.9 ± 0.2	28.0 ± 0.5
Height (relative standard deviation, %)	52	5
τ/σ	0.9 ± 0.1	0.8 ± 0.1
N_{sys} (theoretical plates)	5700 ± 300	6300 ± 300
RSE (relative system efficiency, N_{sys}/N_{max})	0.58 ± 0.07	0.64 ± 0.07

damental index of peak asymmetry), N_{sys} (efficiency of the separation, including chromatographic and non-chromatographic contributions to band broadening), N_{max} (theoretical maximum separation efficiency in the absence of band broadening contributors), and RSE (relative system efficiency, N_{sys}/N_{max}). Simple calculation of τ/σ values is especially useful during separation development. This CFOM provides an objective criterion for assessment of peak shape changes following adjustment of the mobile phase composition.

Peak descriptors and CFOM data obtained from a single chromatographic analysis study are presented in Table II. This analysis consisted of a five-point standard curve and 62 urine samples, all determined in duplicate. The slope of the standard curve was 0.0089, the y-intercept was -0.024 (8% of the chromatographic peak-height ratio at the low point of the standard curve), and the linear regression coefficient was 0.9965. The CFOM calculation algorithm we employed incorporated a sensitive test for distortions of the leading and trailing edges of chromatographic peaks which perturb CFOM calculations. Table II makes note of the small number of peaks rejected for data analysis. Peaks were rejected by the computer program when incompletely resolved from other endogenous sample constituents found in some unusual specimens. The tabulation of capacity factors and retention times (with relative standard deviations of 1.5%) demonstrates the excellent stability of the chromatographic system during experimental trials of as many as 30 h duration. The 52% variation in 3-methylhistidine peak height reflects the large variation of urinary 3-methylhistidine content in this particular analysis series. The 5% variation of 3-ethylhistidine internal standard peak height demonstrates the excellent sample-to-sample reproducibility of the chromatographic procedure.

We have found that τ/σ values of 1–2 are typical in isocratic reversed-phase chromatograms. Greater peak asymmetry is often observed in reversed-phase ion-

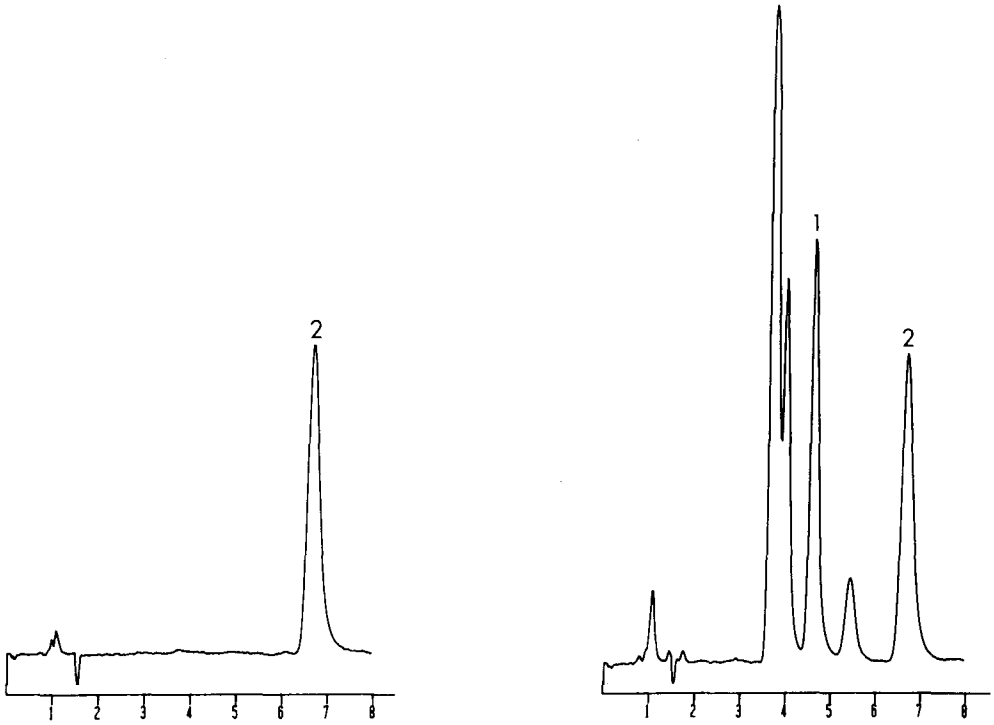

Fig. 1. Chromatogram obtained from a 250-μl aliquot of a 200 nmol/ml solution of the procedural internal standard 3-ethylhistidine carried through the analytical procedure. The column used was a 10×0.8 cm I.D. radially compressed cartridge of Radial-Pak C_{18}, 5 μm particle diameter. The chromatographic eluent was $2.5 \cdot 10^{-2}$ *M* sodium dodecyl sulfate, $3 \cdot 10^{-2}$ *M* sodium phosphate (monobasic) and $2 \cdot 10^{-2}$ *M* 3-(N,N-dimethylamino)-1,2-propanediol in 18% (v/v) acetonitrile–water. The aqueous component was adjusted to pH 3.5 prior to the addition of acetonitrile. The chromatographic eluent was pumped at 4.0 ml/min while the post-column derivatization solution was pumped at 2 ml/min. The fluorometric detector was operated at an excitation wavelength of 240 nm and an emission cut-off filter of 418 nm. The *x*-axis was in min; full-scale deflection (*y*-axis) was 1.6 μA. The injection volume was 3 μl. Peak 2 = 3-ethylhistidine internal standard.

Fig. 2. Representative chromatogram of 3-methylhistidine in human urine using the described procedure. The chromatographic conditions are described under Fig. 1. The injection volume was 3 μl. Peaks: 1 = 3-methylhistidine; 2 = 3-ethylhistidine internal standard. 3-Methylhistidine concentration: 174 nmol/ml.

pair separations of basic and quaternary ammonio compounds [38]. The low τ/σ values (and high RSE values) obtained in this study reflect low contribution to band broadening by both chromatographic and non-chromatographic processes. We noted that during the lifetime of an analytical column, the shape and order of elution of the 3-methylhistidine and 3-ethylhistidine peaks remained constant. However, absolute retention time tended to decrease and column back-pressure at the analytical flow-rate slowly increased. These findings forced replacement of analytical columns after approximately 1500 injections.

Figs. 1 and 2 display chromatograms developed on an analytical column which was near the end of its useful lifetime. Despite this "worst case" chromatographic

column (which accounts for the relatively short separation time), the chromatographic resolution and peak shape remain excellent. Fig. 1 is a chromatogram of the internal standard 3-ethylhistidine carried through the analytical procedure. For this peak, k' is 15.83, τ/σ is 0.95, N_{sys} is 2145 and RSE is 0.52. Fig. 2 is a chromatogram of a representative human urine sample with added internal standard carried through the analytical procedure. The peak descriptors and CFOM for 3-methylhistidine and 3-ethylhistidine are, respectively: $k' = 10.58$ and 15.8, $\tau/\sigma = 1.0$ and 0.91, $N_{sys} = 2442$ and 2357, and RSE = 0.49 and 0.54. Clearly, sensitivity is more than sufficient. The high concentration point of the standard curve (300 nmol/ml) produces a full-scale response of the fluorescence detector with only 1% of the isolated sample injected. The 3-ethylhistidine standard (Fig. 1) shows that the described procedure produces no chromatographic interference with 3-methylhistidine due either to impurities in the 3-ethylhistidine internal standard or the initial small-column ion-exchange sample preparation procedure. In the human urine specimen (Fig. 2), all chromatographic peaks are well formed and clearly resolved, which is reflected in the CFOM data.

We have determined the 3-methylhistidine excretion in a series of metabolic studies involving human fasting, human diabetic ketoacidosis, alcoholic cirrhosis, and following trauma. When seven normal-weight women were placed on a soybean-based liquid complete diet (ENSURE) for two to six days, we observed a urinary excretion of 107 ± 7 μmol 3-methylhistidine per 24 h. This is slightly below the range of the data reported by Monboisse et al. [13] and the six other reports reviewed by those authors. The complete data from our studies will be published separately.

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REFERENCES

1 A.M. Asatoor and M.D. Armstrong, Biochem. Biophys. Res. Commun., 26 (1967) 168.
2 V.R. Young, S.D. Alexis, B.S. Baliga, H.N. Munro and W. Muecke, J. Biol. Chem., 247 (1972) 3592.
3 C.L. Long, L.N. Haverberg, V.R. Young, J.M. Kinney, H.N. Munro and J.W. Geiger, Metabolism, 24 (1975) 929.
4 D.J. Millward, P.C. Bates, G.K. Grimble, J.G. Brown, M. Nathan and M.J. Rennie, Biochem. J., 190 (1980) 225.
5 E.G. Afting, W. Bernhardt, R.W.C. Janzen and H.J. Rothig, Biochem. J., 200 (1981) 449.
6 E. Radha and S.P. Bessman, Anal. Biochem., 121 (1982) 170.
7 M. Elzinga and N. Alonzo, Methods Enzymol., 91 (1983) 8.
8 L.N. Haverberg, H.N. Munro and V.R. Young, Biochem. Biophys. Acta, 371 (1974) 226.
9 D.T. Di Ferrante, N.Y. Wilson and C.S. Leach, J. Chromatogr., 187 (1980) 271.

10 P.W. Emery and M.J. Rennie, Anal. Biochem., 126 (1982) 67.
11 G. Žunić, S. Stanimirović and J. Savić, J. Chromatogr., 311 (1984) 69.
12 R.C. Feldhoff, D.J. Ledden, M.C. Steffen, J.M. Steffen and X.J. Musacchia, J. Chromatogr., 311 (1984) 267.
13 J.C. Monboisse, P. Pierrelee, A. Bisker, V. Pailler, A. Randoux and J.P. Borel, J. Chromatogr., 233 (1982) 355.
14 H. Vielma and J. Mendez, J. Chromatogr., 196 (1980) 166.
15 L. Cotellessa, F. Marcucci, D. Cani, P. Sfondrini, L. Colombo, E. Mussini and F. Poy, J. Chromatogr., 221 (1980) 149.
16 F. Marcucci, L. Colombo and E. Mussini, J. Chromatogr., 336 (1984) 356.
17 D.E. Matthews, J.B. Starren, A.J. Drexler, D.M. Kipnis and D.M. Bier, Anal. Biochem., 110 (1981) 308.
18 F. Marcucci, L. Colombo, G. De Ponte, D. Cani, L. Cobelli, A. Frigerio and E. Mussini, J. Chromatogr., 309 (1984) 132.
19 Z. Friedman, H.W. Smith and W.S. Hancock, J. Chromatogr., 182 (1980) 414.
20 L.C. Ward, M. Miller and S. Hawgood, J. Chromatogr., 223 (1981) 417.
21 W.S. Hancock, D.R.K. Harding and Z. Friedman, J. Chromatogr., 228 (1982) 273.
22 S.S.O. Hung and T.W. Moon, J. Chromatogr., 269 (1983) 114.
23 G. Ali Qureshi, S. Van den Berg, A. Gutierrez and J. Bergström, J. Chromatogr., 297 (1984) 83.
24 P.E. Minkler, E.A. Erdos, S.T. Ingalls, R.L. Griffin and C.L. Hoppel, J. Chromatogr., 380 (1986) 285.
25 J.P. Foley and J.G. Dorsey, Anal. Chem., 55 (1983) 730.
26 E. Grushka, M.N. Myers, P.D. Schettler and J.C. Giddings, Anal. Chem., 41 (1969) 889.
27 E. Grushka, Anal. Chem., 44 (1972) 1733.
28 R.A. Cox and C.L. Hoppel, Biochem. J., 136 (1973) 1083.
29 S. Moore and W.H. Stein, J. Biol. Chem., 211 (1954) 907.
30 A.M. Krstulovic and P.R. Brown, Reverse-Phase High-Performance Liquid Chromatography, Wiley, New York, 1982, p. 214.
31 S.S. Yuan and A.M. Ajami, J. Labelled Compd. Radiopharm., 21 (1984) 97.
32 B.A. Bidlingmeyer and F.V. Warren, Anal. Chem., 56 (1984) 1584A.
33 J.J. Kirkland, W.W. Yau, H.J. Stoklosa and C.H. Dilks, J. Chromatogr. Sci., 15 (1977) 303.
34 H.M. Gladney, B.F. Dowden and J.D. Swalen, Anal. Chem., 41 (1969) 883.
35 R.E. Pauls and L.B. Rogers, Anal. Chem., 49 (1977) 625.
36 S.N. Chesler and S.P. Cram, Anal. Chem., 43 (1971) 1922.
37 W.W. Yau, Anal. Chem., 49 (1977) 395.
38 P.J. Twitchett and A.C. Moffat, J. Chromatogr., 111 (1975) 149.