High-performance liquid chromatographic estimation of carbamylated amino acids

V. K. Sharma, G. J. Rao, R. K. Jadhav, Heeresh Chandra and S. Sriramachari*
Medicolegal Institute, Gandhi Medical College Building, Bhopal 462 001, India
*Institute of Pathology, Safdarjang Hospital, New Delhi 110 029, India

A method for the estimation of carbamylated amino acids in terms of their respective hydantoins has been developed on high-performance liquid chromatography using an ultraviolet spectrophotometric detector. The detection limit of the method was noted to be 10 ng for methylvalinehydantoin (MVH). The amino acids like glycine, threonine and methionine were treated with methyl isocynanate to prepare standard hydantoins while valine was treated with methyl isocyanate as well as KCNO to make methylated and non-methylated hydantoins respectively.

CYANATES react with primary amino groups to form either N-substituted amides of carbamic acid or ureas, if vicinal carboxylic acid group is present (as occurs in L-amino acid hydrolysates of polypeptides that have been treated with a cyanate) then a 'hydantoin' may be formed. Cyanates may be inorganic or organic and may be in the form of isocyanate also[1].

The commonly used method for determination of hydantoins in biological materials involves the formation of hydantoins from carbamylated amino acid and subsequent identification by gas chromatography. This method has been used to analyse the blood samples from patients with sickle-cell disease, who have been administered cyanates either orally or intravenously, to monitor the haemoglobin carbamylation level.

We have not come across any method, in the available literature, for the identification of methylated hydantoins of amino acids by high-performance liquid chromatography. This paper reports the sensitive HPLC method for the identification of the same.

Standard methylated and non-methylated hydantoins of various amino acids like valine, phenyl alanine, threonine, methionine and glycine were prepared following the method described by Manning *et al.*[2] The formation of these hydantoins was confirmed by IR and mass spectra. *In vitro* studies were also made by adding carbamylating agents like KCNO and methyl isocyanate to the blood and other body tissues.

The hydantoins were chromatographed on Zorbax ODS Column (4.6 × 25 cm). HPLC equipment (Shimadzu, Japan, model LC-6A) consisted of two pumps, a system controller SCL-6A UV-Vis spectrophotometric detector SPD-6-AV and a CR-3A computing integrator. The chromatographic analysis was performed at ambient temperature and at a flow rate of 1 ml/min. The mobile phase consisted of water methanol (80 : 20). The injected sample volume was 20 μl of aqueous solution of hydantoins. The detector wavelength was set at 210 nm, since their spectroscopic study revealed that these hydantoins show absorption maxima in ultraviolet region suitably at 210 nm wavelength. Total procedure time was 30 min.

A good separation was obtained in the hydantoins of various amino acids (Figure 1). The order of the elution of the peaks of methylated hydantoins was glycine, phenylalanine, threonine, valine and methionine. In non-methylated hydantoins only valine was studied which elutes after glycine.

To achieve this separation mobile phase composition was repeatedly changed and best separation was noticed when water–methanol were used in the ratio of 80 : 20.

The detection limit of the method is in nanograms. It was noted to be 10 ng for methylvalinehydantoin. Detector response was observed to be linear for MVH concentration (Figure 2). Values of area counts recorded were the mean of four time analysis of each concentration. Ethyl acetate extracts of blood and tissue samples

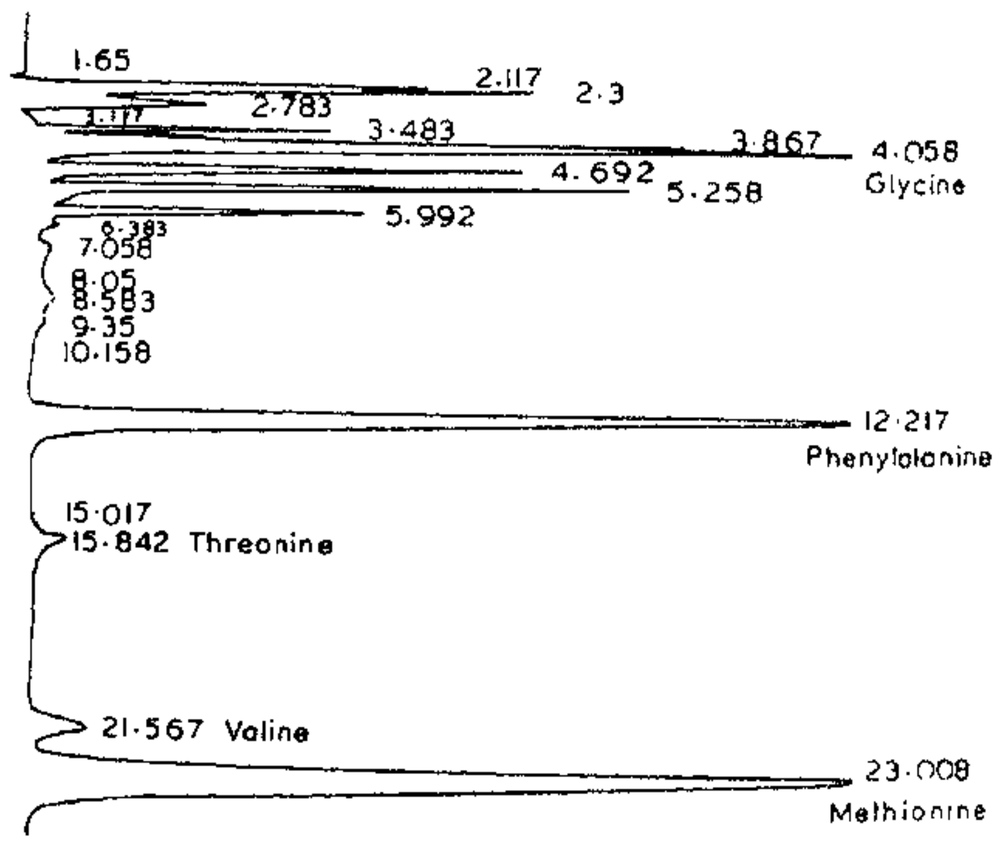

Figure 1. Methylated hydantoins of amino acids.

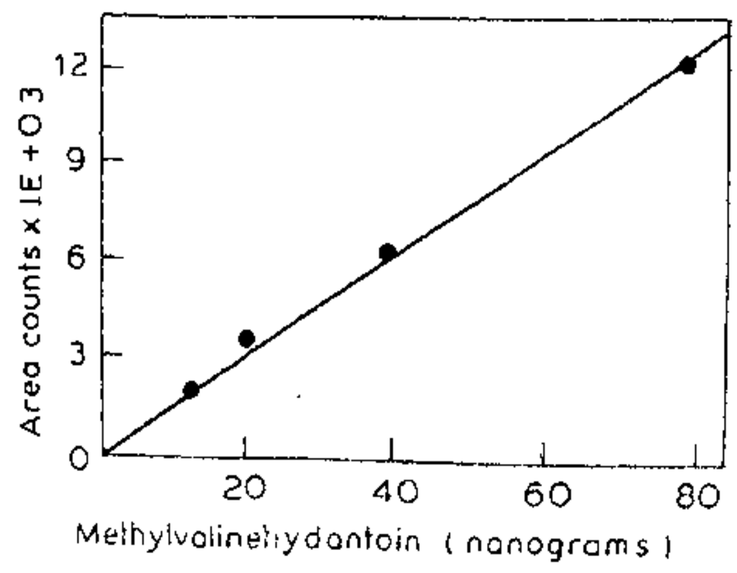

Figure 2. Linear curve plotted as area counts vs MVH concentration.

Table 1. Recovery of methylvalinehydantoin from various body tissues.

Tissue	Amount added in μg/g of tissue	Present HPLC method		Manning *et al.* GC method	
		Amount recovered (μg*)	Recovery (%)	Amount recovered (μg*)	Recovery (%)
Blood	5	4.50	90	4.55	91
	10	8.90	89	9.00	90
	20	18.50	92.5	18.40	92
Lung	5	4.30	86	4.35	87
	10	8.50	85	8.50	85
	20	17.90	89.5	18.00	90
Brain	5	4.25	85	4.20	84
	10	8.55	85.5	8.60	86
	20	18.10	90.5	18.20	91

*Mean values of the replicate of three sets.

were also studied after stripping the solvent under reduced pressure and dissolving the dried extracts in 4% acetonitryl water. Recovery studies were carried out by adding known amount of methylvalinehydantoin in varying concentrations to the blood, lung and brain tissue in three sets. It was found to be 85–92%, which agrees with the results of Manning *et al.*[2] (Table 1). Reproducibility of the results was always found to be more than 90%.

The carbamylation of NH_2-terminal amino acids of proteins alters their functional properties as oxygen affinity in the case of haemoglobin[3]. For sequencing of proteins cyanates are the favourable reagents. Therefore, biological application of this method is quite significant especially for the determination of carbamylation level in body tissues and fluids, in the cases of sickle-cell anaemia and the cases exposed to some cyanates and isocyanates like the victims of the Bhopal Gas Disaster who are supposed to have been exposed to methyl isocyanate and other gases.

1. Cohen, S. and Oppenheimer, E., in *The Chemistry of Cyanates and their thio derivatives* (ed. Saul Patai), John Wiley and Sons, New York, (part II, 1977).
2. Manning, J. M., Lee, K. C., Ceremi, A. and Gillette, P. N., *J. Lab. Clin. Med.*, 1973, **81**, 941.
3. Jensen, M., Nathan, D. G. and Bunn, H. F., *J. Biol. Chem.*, 1973, **218**, 8057.

ACKNOWLEDGEMENT. We thank Dr P. K. Ramchandran, Former Director, Defence Research and Development Establishment, Gwalior, for extending help in preparation and characterization of the standard hydantoins of amino acids.

16 March 1989