ELSEVIER

Journal of Chromatography B, 696 (1997) 117–122

JOURNAL OF CHROMATOGRAPHY B

Use of the ASTED system to determine L-N^G-monomethylarginine (546C88) in human plasma by pre-column *o*-phthalaldehyde derivatisation and high-performance liquid chromatography

J.D.H. Cooper[a,*], N. Shearsby[a], C.T.C. Fook Sheung[b]

[a]*Clinical Innovations Ltd., Building 45, Stoneleigh Deer Park, Stareton, Kenilworth, Warwickshire CV8 2LY, UK*
[b]*Department of Bioanalysis and Drug Metabolism, Glaxo Wellcome plc, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, UK*

Received 15 October 1996; revised 30 January 1997; accepted 30 January 1997

Abstract

The use of the system automated sequential trace enrichment of dialysates (ASTED), to prepare plasma samples for the estimation of L-N^G-monomethylarginine (546C88) by pre-column *o*-phthalaldehyde–2-mercaptoethanol derivatisation and HPLC is described. Calibration is achieved using purified albumin as a substitute matrix for plasma. Using this technique the procedure was observed to be specific for 546C88 and linear over the range 0.10 to 50.0 μmol/l. The within-run imprecision (C.V.) at four different spiked plasma 546C88 concentrations of 0.10, 1.0, 8.0 and 40.0 μmol/l was 6.48, 2.55, 2.79 and 3.37%, respectively, and the between-run imprecision (C.V.) estimated to be 8.50, 1.80, 2.10 and 3.30%, respectively, for the same spiked 546C88 concentrations. The overall accuracy (% bias) of the procedure using an albumin matrix for calibration was estimated to be −2.50, −5.25, −3.56, −3.53%, respectively, and the recovery of 546C88 from six different spiked plasma samples estimated to be 99.1±1.4%.

Keywords: L-N^G-Monomethylarginine

1. Introduction

L-N^G-Monomethylarginine (546C88) is a competitive inhibitor of nitric oxide synthase isoenzymes which catalyse the formation of nitric oxide from the amino acid L-arginine in a variety of cell types. Nitric oxide is recognised as a potent vasodilator and high levels observed in patients with septic shock may play a key role in the subsequent development of multiple organ dysfunction and death that is typical of severe sepsis. 546C88 may therefore offer an advance in the treatment of septic shock by inhibiting excess nitric oxide formation preventing its associated vasodilation and tissue damage. A means to determine plasma 546C88 concentrations is therefore essential for pharmacokinetic investigations of the compound during clinical studies.

Since the introduction of the fluorescent reagent, *o*-phthalaldehyde–2-mercaptoethanol (OPA–MCE), by Roth [1] numerous publications have appeared using its properties for pre-column preparation of amino acid fluorophore derivatives (1-alkylthio-2-alkylisoindoles) and their separation using HPLC [2–5]. Since 546C88 is a metabolite of a primary

*Corresponding author.

PII S0378-4347(97)00104-7

amino acid, reaction with OPA–MCE will yield the highly fluorescent OPA–MCE–546C88 adduct enabling good assay specificity and analysis of small sample volumes.

Most biological sample (e.g., plasma) preparation procedures prior to OPA–MCE derivatisation have been performed manually utilising sulphosalicylic acid protein precipitation. Such procedures are tedious since the pH optimum of the OPA–MCE reaction is >9.0 and many operational tasks are required before the prepared extract is suitable for injection onto the HPLC. Early ASTED (automated sequential trace enrichment of dialysates) applications concerned the analysis of amino acids using pre-column derivatisation with OPA–MCE [6]. This technique has the advantage of removing protein in an efficacious manner without resorting to acid denaturants.

The method developed for the automatic estimation of plasma 546C88 utilises an ASTED configuration with increased sample preparation capabilities previously described in earlier publications [7,8] and reversed-phase gradient HPLC for the separation of the fluorescent isoindole product. In this manner plasma is automatically mixed with a reference amino acid, homoarginine (HARG) followed by OPA–MCE. The resulting derivatives are dialysed and enriched prior to injection onto the HPLC column.

This paper reports the development of an ASTED method for the determination of 546C88 in human plasma. 546C88 is physiologically present in human plasma and to negate calibration difficulties the use of a purified albumin solution as a substitute matrix is investigated.

2. Experimental

2.1. Instrumentation

Unless otherwise stated all of the equipment in the HPLC and ASTED units was obtained from Anachem (Luton, UK).

2.1.1. HPLC

The gradient HPLC system consisted of Models 306 and 307/5SC pumps, an ABI FS980 fluorescence detector and a Rheodyne 7010 injection valve fitted on the ASTED unit. Control of the HPLC system, integration of chromatographic peaks and communication with the ASTED system (via Gilson Medical Electronics GSIOC) was made using a 715, V1.2, system controller (IBM PS1 with hard disc, EGA graphic card, mouse, MS DOS and Windows software V3.1).

2.1.2. Sample preparation (ASTED) unit

The software and hardware modifications to an ASTED version A unit to enhance its capabilities (e.g., "heart-cutting") have been described previously [7]. The modified ASTED unit basically comprised a 231 auto-sampling injector; two 401 dilutors fitted with 1 ml syringes (controlling sample pretreatment on the donor side and dialysate flow on the recipient side of the dialyser); a universal valve switching module to isolate the dialyser unit from the trace enrichment device to enable "heart-cutting" routines; a Kel F dialyser unit with a 370 μl donor volume fitted with a 15 kD Cuprophan membrane and a stainless-steel trace enrichment cartridge (Prelute, Anachem) packed with 70 mg of 10 μm Hypersil ODS (Shandon Southern Products, Runcorn, UK) to replace the loop on the Rheodyne 7010 injection valve. Control of the ASTED operations was made using Process 4 V1.02 software (Clinical Innovations, Kenilworth, UK).

2.2. Reagents

2.2.1. General reagents

Unless otherwise stated, all chemicals were analytical grade obtained from Sigma (Poole, UK). HPLC grade water, prepared using a Purite (Thame, UK) system, was used for all reagent preparations. HPLC solvents were obtained from Romil (Cambridge, UK). The following stock reagents were prepared: dipotassium hydrogen orthophosphate (DKHP) buffer (500 mmol/l, pH 7.0 and pH 5.0); borate buffer (100 mmol/l, pH 9.5); OPA–MCE reagent (22 mmol/l: 75 mg dissolved in 2 ml methanol, made to 25 ml with borate buffer and 75 μl of MCE added); 50 g/l bovine albumin solution (Sigma grade V) dissolved in 0.05% sodium azide; HARG solution (0.25 μmol/l in water). Water and 20 mmol/l DKHP buffer (pH 7.0) were dispensed by

the Gilson 401 dilutors to respectively purge the donor and recipient channels and connections of the dialyser unit.

2.2.2. Standard preparations

546C88 (hydrochloride salt) was obtained from Wellcome Research Laboratories (Beckenham, UK). A 500 μmol/l stock solution of 546C88 in water was prepared. Working standards (25.0 and 100.0 μmol/l) were prepared by diluting the stock solution in water. Calibration standards (ranging from 0.10 to 50.0 μmol/l) were prepared fresh by diluting working standards with albumin solution. All standard preparations contained 0.05% (w/v) sodium azide as preservative.

2.3. Chromatographic conditions

A stepwise gradient elution mobile phase (degassed before use) was used to optimise the chromatography conditions. The gradient mobile phase consisted of solvent A [acetonitrile–DKHP (pH 5.8)–water–dimethylsulphoxide (9:10:71:10, v/v)] and solvent B [acetonitrile–water–dimethylsulphoxide (50:40:10, v/v)]. The gradient conditions were at time 0 min 2.5% B; at 10 min 0% B; at 10.1 min 100% B; at 11.0 min 100% B; at 11.1 min 0% B; at 16 min loop to time 0 min; the flow-rate was 1.5 ml/min.

The HPLC column (100×4.6 mm I.D.). was packed with 5 μm particles of Spherisorb ODS2 (Technicol, Stockport, UK). No guard column was employed and the analytical column run at ambient temperature. The detector was set at 240 nm wavelength excitation with a 418 nm cut-off filter for emission.

2.4. Sample preparation

Sample preparation was carried out in a completely automated manner using the ASTED system and optimised as follows: 20 μl of plasma was mixed with 200 μl of HARG solution, 200 μl of OPA–MCE reagent and 400 μl of this mixture delivered to the donor channel of the dialyser. A 3500 μl volume of recipient solvent moved through to the Prelute cartridge over 5 min. Following enrichment, 200 μl of 10% acetonitrile in water (v/v) followed by 1000 μl of water via the 401 dilutor attached to the needle injection unit of the ASTED system, was passed through the Prelute cartridge. The Rheodyne 7010 valve was then switched to INJECT for 1 min and then returned to the LOAD position. A 200 μl volume of 80% tetrahydrofuran in water (v/v) followed by 1000 μl of water was dispensed through the Prelute cartridge via the injection port. The system was then purged and the Prelute cartridge regenerated with 500 μl of recipient solvent ready for the next sample.

2.5. Quantification

Calibration standards were situated at the beginning and end of each analytical run. A linear regression (weighting $1/X^2$) was performed on the peak areas and concentrations of both sets of standards. The regression line established was used to calculate test analyte concentrations.

2.6. Quality control

Heparinised plasma samples (obtained from healthy volunteers) were supplemented with 0.10, 1.0, 8.0 and 40.0 μmol/l of 546C88. These samples were aliquoted and stored at −20°C together with the basal heparinised plasma. The 546C88 aqueous solutions used to supplement the plasma were prepared from separate weighings to those solutions used to prepare the calibration standards.

3. Results

3.1. Optimisation of sample preparation and chromatography conditions

Initial chromatographic conditions were established using gradient elution with the sample preparation conditions described. Figs. 1 and 2 show the chromatograms obtained using this method to analyse the basal heparanised plasma sample and a plasma sample supplemented with 8.0 μmol/l of 546C88, respectively. No chromatographic interferences from OPA–MCE derivatives of the amino acids arginine, alanine, 3-methylhistidine and taurine were observed. Based on previous publications [2]

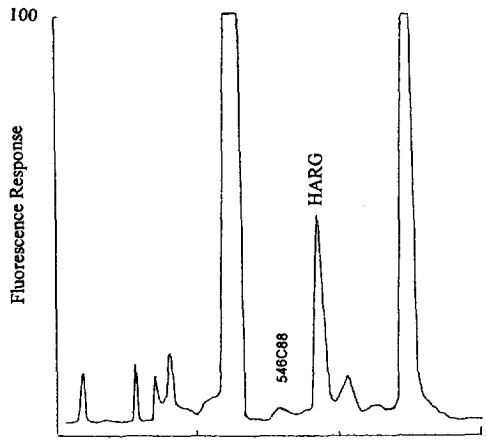

Fig. 1. Chromatogram of human plasma with a basal 546C88 concentration of 0.16 μmol/l.

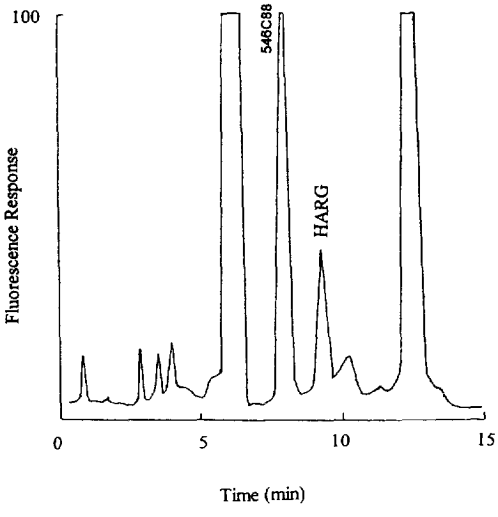

Fig. 2. Chromatogram of human plasma spiked with 8.0 μmol/l 546C88.

these derivatives would elute with similar retention times to the 546C88 derivative.

3.2. Assay performance

3.2.1. Linearity and limits of quantification

Peak areas varied linearly over the analytical range employed. The basal plasma 546C88 concentration ranged from 0.11 to 0.27 μmol/l obtained from measuring six human plasma samples obtained from healthy volunteers and the quantification detection limit was set at the lowest standard concentration on the calibration curve.

3.2.2. Imprecision

The within-run coefficient of variation (C.V.±%) was estimated by assaying the quality control samples ten times in the same analytical run. The between-run (C.V.±%) was obtained by estimating six replicates each of the same control samples in a further three analytical runs. The results are shown in Table 1.

3.2.3. Accuracy

The overall % bias of the of the assay was obtained by measuring six replicates of the basal plasma 546C88 concentrations in the same four analytical runs used to derive the between-run imprecision and subtracting the measured mean concentration from the measured mean plasma 546C88 concentration in the quality control samples. These results are shown in Table 1.

3.2.4. Matrix effects

To test for matrix differences between specimens the relative recovery of the method was obtained by supplementing six different plasma samples with a

Table 1
Within- and between-run imprecision and overall accuracy of the ASTED plasma 546C88 method

Nominal concentration (μmol/l)	Mean overall measured concentration (μmol/l)	Within-run imprecision (C.V., %)	Between-run imprecision (C.V., %)	Overall inaccuracy (% bias)
0 (basal)	0.13			
0.1	0.23	6.48	8.50	−2.50
1.0	1.08	2.55	1.80	−5.25
8.0	7.86	2.79	2.10	−3.56
40.0	38.70	3.37	3.30	−3.53

nominal 546C88 concentration of 40.0 μmol/l and analysing both the supplemented and basal plasma samples using the method described. The basal 546C88 concentrations ranged from 0.10 to 0.17 μmol/l. After subtracting the mean basal 546C88 from the total estimated 546C88 concentration the relative recovery was found to be 99.1±1.4%.

4. Discussion

4.1. Chromatographic separation

The detection of physiological amino acids and their metabolites requires some form of derivatisation. The relatively low polarities of the OPA–MCE fluorophores are ideally suited to pre-column methodology and reversed-phase HPLC to promote rapid chromatographic separations. The detection and quantification of low concentrations of any one amino acid or metabolite may be difficult considering the high physiological plasma concentrations of many potential chromatographic neighbours. This was the case for the estimation of 546C88 in plasma at basal concentrations (approximately 0.10 μmol/l) compared with the relatively close elution time of arginine which may be present at physiological plasma concentrations of 100 μmol/l. In order to obtain sufficient chromatographic space for accurate quantification of low 546C88 plasma concentrations the mobile phase required the inclusion of dimethyl sulphoxide (Figs. 1 and 2). To assess chromatographic separations and sampling efficacy a reference compound homoarginine was automatically added to samples during derivatisation. This compound was not used for quantification purposes.

4.2. Sample preparation

The use of OPA–MCE to derivatise amino acids is a well established technique and the high quantum fluorescent yields ensure that small sample volumes can be analysed. A previous report [9] using the ASTED technique has demonstrated that amino acids can be derivatised by OPA–MCE in plasma directly and that zero order conditions are observed with respect to the OPA–MCE concentration. This procedure has readily been adapted to the ASTED system for the estimation of the amino acid metabolite, 546C88, in human plasma. Following OPA–MCE derivative formation the fluorophores readily diffused across a cuprophan membrane into phosphate buffer and the non-polar OPA–MCE analytes in the dialysate enriched onto silica C_{18} material and are eluted by the HPLC mobile phase during injection.

The non polar nature of the OPA–MCE 546C88 derivative ensured that the breakthrough volume of the compound for the enrichment material was in excess of 8.0 ml. Although the pH of the OPA–MCE reaction is >9.0 the phosphate buffer solution, as the recipient of the dialysate, ensures no solubilisation of the silica enrichment material and as a consequence several hundred sample cycles can be achieved using a single trace enrichment device.

Discussions concerning the importance of sample preparation and the attributes of on-line systems such as ASTED have been previously published [10]. The efficacy of the membrane to remove protein and particulate matter that can interfere chromatographically and reduce column lifetimes has been enhanced by the improved specificities obtained using a "heart-cutting" approach on the material used to enrich the dialysates. This has previously been described and utilises specialised software control extending the analytical capabilities of the basic ASTED system [7] although since its conception a commercial version of the process (ASTED XL) has been made available by Gilson Medical Electronics. This version has also operated equally well to estimate 546C88 in plasma. This process enabled elimination of the highly non polar OPA–MCE amino acid derivatives (e.g., with dibasic functional groups) that would not be eluted during injection and would cause interferences during subsequent chromatography separations. The removal of these potential interferences was accomplished by washing the trace enrichment material with a high strength organic phase prior to preparing the next sample. The net effect of this approach is to reduce the analysis time and combined with the concurrent sequential process of preparing a sample during the chromatography of the previous specimen, increase sample throughput markedly.

4.3. Calibration of the assay

546C88 is naturally present in human plasma and difficulties arise with calibrating any analytical procedures where the compound is present in the matrix under investigation. This was the case for 546C88 especially when there was a need to accurately estimate plasma basal levels of the compound in subjects with severe pathological disturbances. During this investigation the basal 546C88 concentrations ranged from 0.10 to 0.17 μmol/l in six different plasma samples and is in agreement with a previous publication [11]. It has been demonstrated [9] that amino acids, with the exception of tryptophan, are not protein bound and that with the pre-column OPA–MCE conditions used, no such matrix effects occurred for 546C88 in plasma with the ASTED approach. Other alternative matrices, which do not contain 546C88, could then be adopted for calibration purposes. With this ASTED method for analysing 546C88 in plasma, albumin was used as the matrix material to mimic possible viscosity and Donnan effects [12] that may occur when analysing plasma samples for organic molecules that are ionisable. Results to justify this approach are shown in Table 1 where the overall accuracy (% bias) of the method was linear over the quality control 546C88 concentration range tested. At this time the optimum 546C88 loading required is uncertain and as a consequence the plasma calibration range selected is based on previous studies. The pharmacokinetics of 546C88 at different doses and its associated clinical effects in man is, at present, undergoing evaluation. Further work has utilised this approach for the estimation of 546C88 in urine. Assay conditions were exactly the same as the plasma procedure described except that ammonium phosphate was used as the matrix for the calibration standards.

4.4. Sample preparation and matrix effects

Many procedures can suffer from the effects of varying matrices and previous reports using the ASTED procedure have recognised this fact [10]. The relative recovery of 546C88 from various samples would suggest that between-sample variations are not problematical.

4.5. Assay performance

The use of this technique has provided rapid means to examine the pharmacokinetics of 546C88 and the acceptable assay performance (Table 1) has been consistent during the analysis of many hundreds of plasma samples over a two year period. Furthermore, the procedure has operated with a similar assay performance on the more recent Gilson ASTED XL system.

References

[1] M. Roth, Anal. Chem., 43 (1971) 880.
[2] D.C. Turnell and J.D.H. Cooper, Clin. Chem., 28 (1982) 527.
[3] M. Griffin, S.J. Price and T. Palmer, Clin. Chim. Acta, 125 (1982) 89.
[4] D.L. Hogan, K.L. Kraemer and J.I. Isenberg, Anal. Biochem., 127 (1982) 17.
[5] J.C. Hodgin, J. Liq. Chromatogr., 2 (1979) 1059.
[6] J.D.H. Cooper and D.C. Turnell, J. Auto. Chem., 7 (1985) 181.
[7] J.D.H. Cooper, A.R. Buick and C.T.C. Fook Sheung, Lab. Robo. Autom., 6 (1994) 15.
[8] J.D.H. Cooper, C.T.C. Fook Sheung and A.R. Buick, J. Chromatogr. B, 652 (1994) 15.
[9] J.D.H. Cooper, Chromatography Society Symposium, Plenum Publishers, London, 1992, p. 87.
[10] J.D.H. Cooper, D.C. Turnell, B. Green and F. Verillon, J. Chromatogr., 456 (1988) 53.
[11] R.J. Macallister, S.A. Fickling, G.S.J. Whitley and P. Vallance, Br. J. Pharmacol., 112 (1994) 43.
[12] F.G. Donnan, Chem. Rev., 1 (1924) 73.