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Short communication

Determination of derivatized amino acids in human embryo culture media by gas chromatography

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Abstract

An adequate analytical method for determination of amino acids can provide a better insight in the metabolism of *in vitro* human embryo cultures, increasing the success rate of embryo implantation. Since individual amino acid amounts per embryo occur in the nanogram range, GC was the technique of choice, due to its inherent sensitivity and high sample throughput. Amino acids were analyzed as alkyl formate derivatives. The limits of detection (LOD) of all amino acids involved were in the sub-nmol range. The high risk of sample contamination proved to be the major analytical issue, but it could be overcome. For an extended method sensitivity, a simple preconcentration step could also be used.

Keywords: Amino acids; Embryo; Derivatization; Gas chromatography

1. Introduction

The success of implantation of artificially fertilized human embryos has increased in the past years, since the knowledge in predicting the developmental fate of single embryos has also increased. The current methods for predicting embryo development to the blastocyst stage and selecting proper embryos for implantation are mostly based on morphological criteria [1]. However, the success of implantation based on these criteria is nowadays only 10–30% [2]. It has been demonstrated that morphology is a poor predictor of embryo viability [3]. Moreover, the most distinctive differences between viable and non-viable embryos were observed in their amino acid turnover [4]. Finding an optimized amino acid composition of culture media would improve *in vitro* embryo metabolism and consequently increase the possibilities of implantation. The optimization of the amino acid composition of culture media requires several single-embryo cultures from various developmental phases to be analyzed in order to obtain the profile of amino acid turnover throughout the embryo development.

Previous work on the determination of amino acid turnover in embryo culture media [1,4] was based on HPLC with fluorescence detection [5]. In comparison to HPLC, GC provided higher sample throughput and a more cost-effective approach, using the EZ:faast^{TM} proprietary sample preparation and analysis procedure [6], although very polar amino acids (e.g. ARG) could not be analyzed with this procedure. In principle, the mentioned procedure involves a selective μSPE, a previously known amino acid derivatization chemistry using an alkyl chloroformate reagent [7] and an optimized GC separation with a dedicated column stationary phase. The utility of this analytical procedure in monitoring physiological amino acid levels has been already demonstrated [8,9].

Due to the selectivity of the method and the embryo culture media as a relatively simple sample matrix, flame ionization detection (FID) was successfully employed instead of a more costly MS detection. In this study, 15 essential amino acids were evaluated for their turnover in embryo culture media. The main issues represented sample handling of small sample volumes (4 μL) and the prevention of sample contamination, since the individual amino acid amounts in the sample were in

Abbreviation: μSPE, micro-solid phase extraction

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the nanogram range. A simple sample reconcentration step for improved sensitivity is also discussed.

2. Materials and methods

2.1. Instrumentation

The analyses were performed using a gas chromatograph Finnigan Focus GC with FID, and equipped with an AI3000 autoinjector for liquid samples (Thermo, Rodano Milan, Italy) and data acquisition software ChromCard version 2.0 (Thermo). The column in use was Zebron™ ZB-AAA with dimensions 10 m × 0.25 mm i.d. and a proprietary stationary phase (Phenomenex, Torrance, CA, USA). The analytical conditions are given in Table 1.

2.2. Chemicals and materials

Isooctane and phosphorous(V) oxide (Merck, Darmstadt, Germany) were of analytical grade. Bidistilled water was obtained from a Megapure® apparatus (Barnstead, Dubuque, IA, USA). The EZ:faast™ sample preparation kit was supplied by Phenomenex. Culture media of human embryos (4 μL each) were obtained by carefully separating the *in vitro* fertilized developing embryo from its surrounding medium and transferring the corresponding liquid for further analysis. Bulk culture medium was purchased from MediCult (Jyllinge, Denmark). For pipetting, sterile pipette tips were used (Biohit, Helsinki, Finland).

2.3. Glassware handling

The laboratory glassware used in the sample preparation procedure, including vials and Pasteur pipettes, was autoclaved at 121 °C for 1 h.

2.4. Preparation of standards

The working standard solution was prepared by transferring 25 μL of the corresponding amino acid standard stock solutions (containing 5 nmol of each amino acid) in the sample preparation vial and prepared according to the EZ:faast™ procedure.

One hundred microliters of Reagent 1 (internal standard, norvaline) were added to the sample aliquot and vortexed. The resulting solution was extracted in the sorbent of the μSPE tip and the remaining liquid discarded. Two hundred microliters of Reagent 2 were then used to wash the sorbent. The remaining liquid in the μSPE tip was removed by pulling air through it. After that, the amino acids in the μSPE tip were extracted in 200 μL of Eluting Medium (Reagent 3A/Reagent 3B 3:2, v/v). The extract was derivatized by adding 50 μL of Reagent 4 and emulsifying the mixture. One hundred microliters of Reagent 5 (organic solvent) were then added and the mixture was re-emulsified. The organic and aqueous phase of the emulsion were separated by acidifying it with 100 μL of Reagent 6 and vortexing. The upper, organic layer was transferred to a GC vial using a Pasteur pipette and used for analysis.

For sensitivity and linearity tests, the volumes of amino acid standard stock solutions used for derivatization ranged from 1 μL to 100 μL and prepared according to the mentioned procedure.

Table 1
GC conditions

Carrier gas	Helium
Flow mode	Constant flow, 1.5 mL/min
Split ratio	1:15
Injection	2.0 μL, needle preheating 4 s
Inlet temperature	250 °C
Detector temperature	320 °C
GC column	ZB-AAA (Zebron™, Phenomenex), 10 m × 0.25 mm
Oven temperature program	110–320 °C at 32 °C/min, run time 6.56 min

2.5. Sample preparation

Four microliters of culture media from individual embryos or bulk culture medium were diluted with 200 μL of bidistilled water. After vortexing, 150 μL sample aliquots were prepared in the same way as standards.

For accuracy tests, 150 μL sample aliquots were used with and without the addition of 25 μL of the corresponding amino acid standard stock solutions, and prepared according to the mentioned procedure.

2.6. Sample reconcentration

Sample solutions containing low amounts of analytes were dried under a gentle stream of dry nitrogen and then redissolved in 35 μL of isooctane. Sample solutions with water present in the organic phase (see below) were dried under a stream of nitrogen first and the remaining traces of water were removed by dessication over phosphorous(V) oxide overnight. The dry residue was then redissolved in 35 μL of isooctane.

2.7. Method precision, accuracy, sensitivity and linearity

Precision was determined by injecting six replicates of standard solution containing 5 nmol of each amino acid of interest. Accuracy was determined as a mean recovery from standard additions of 5 nmol of each amino acid to bulk culture medium samples in six replicates. Repeatability was determined by preparing six replicates of bulk culture medium. Linearity was checked in three replicates in the range between the limit of quantitation (LOQ) and 20 nmol of each amino acid, at least in six points. Correlation coefficients were calculated with intercept values set at zero. All calculations were based on the amino acid/internal standard peak area ratios. The determination of the limits of detection (LOD) and quantitation (LOQ) was based on the signal-to-noise ratios of 3 and 10, respectively. The validation parameters were obtained without sample reconcentration and are shown in Table 2. Examples of standard and sample chromatograms are shown in Fig. 1A and B, respectively.

Table 2
Validation parameters of the method

Parameter Amino acid	Injection precision (%R.S.D., $n=6$)	Accuracy (%)[a,b]	Repeatability (%R.S.D., $n=6$)[b]	LOD (nmol)[c]	LOQ (nmol)[c]	Linear range (nmol)[c]	Regression coefficient (r)[d]
ALA	0.5	101.3	1.2	0.3	1.0	1.0–20.0	0.9965
LEU	0.4	105.1	1.6	0.2	0.6	0.6–20.0	0.9983
ILE	0.6	101.5	1.2	0.2	0.7	0.7–20.0	0.9960
THR	0.8	93.5	2.3	0.3	1.0	1.0–20.0	0.9959
SER	1.1	99.2	2.0	0.4	1.3	1.3–20.0	0.9945
PRO	0.4	94.2	1.7	0.2	0.7	0.7–20.0	0.9961
ASN	1.3	90.4	2.1	0.3	1.1	1.1–20.0	0.9974
ASP	1.0	95.5	1.3	0.3	0.9	0.9–20.0	0.9965
MET	1.4	94.4	0.8	0.2	0.8	0.8–20.0	0.9975
GLU	2.3	94.4	2.6	0.7	2.3	2.3–20.0	0.9971
PHE	1.2	101.0	0.8	0.2	0.5	0.5–20.0	0.9986
GLN	0.9	91.7	5.5	0.8	2.5	2.5–20.0	0.9962
LYS	1.1	116.7	2.8	0.4	1.2	1.2–20.0	0.9973
TYR	1.4	100.1	2.3	0.2	0.7	0.7–20.0	0.9962
TRP	1.2	105.9	1.7	0.2	0.6	0.6–20.0	0.9952

The results are obtained without sample reconcentration. LOD: limit of detection; LOQ: limit of quantitation.
[a] Determined by sample spiking.
[b] Determined on bulk culture medium.
[c] Refers to the amount of analyte present in the sample.
[d] Intercept values are set at zero.

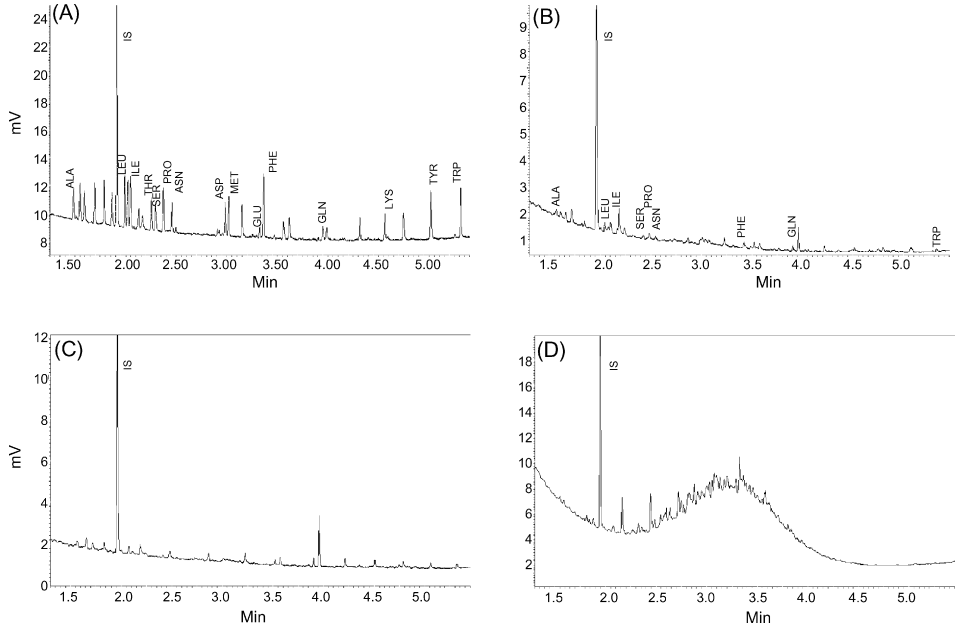

Fig. 1. Gas chromatograms from (A) standard solution; (B) sample solution; (C) blank sample solution prepared with contaminated water; (D) sample solution containing water emulsion. IS, internal standard (norvaline).

3. Results and discussion

3.1. Analytical procedure

Alkyl formylation of amino acids according to the EZ:faast^{TM} procedure [6] proved to be a useful means for amino acid analysis in embryo culture media due to its simplicity and high-throughput in both sample preparation and chromatographic separation. In contrast, a commonly used HPLC procedure for this type of application [5] takes about five times more in terms of analytical run time.

Despite the simplicity of sample preparation, there were some analytical issues, though. The main issue was the prevention of sample contamination, providing labware cleanliness by a proper equipment sterilization. Water used for sample preparation was also an important parameter. As evidenced in Fig. 1C, contaminated water could give misleading results, due to the artifacts present in the chromatogram. Rigorous labware sterilization and the use of freshly prepared water overcame this problem. Another problem resulted from the presence of a fine water emulsion in the final sample solution. In such a case, peak quantification was virtually impossible, due to the alterations in

the baseline and in peak retention times (Fig. 1D). The most feasible explanation for such artifacts in the chromatogram is the occurrence of solvent effects or even of stationary phase stripping [10], since the presence of water significantly contributes in increasing the polarity of the solvent injected. However, the latter problem occurred in only about 15% of all samples analyzed and it was readily solved by applying sample reconcentration described in Section 2.6.

Although the expected gain in sensitivity with sample reconcentration should be higher, since the solvent volume is reduced by two thirds, the increase in sensitivity was about 2.1-fold for virtually all the amino acids involved in the analysis. Sample reconcentration provided a useful way to obtain more reliable data from low amino acid concentrations, but the difference between the theoretical and the experimental gain in sensitivity indicates a significant presence of chemical noise originating from the sample solution. Whether the noise originates from the sample matrix or from the derivatization reagents, a detailed investigation on the problem should be done.

3.2. Sample analyses

About 200 culture media samples from different embryo developmental phases were analyzed. The content of various amino acids varied from below 0.1 nmol to over 10 nmol per 4 μL of culture medium. However, amino acid contents lower than 0.3 nmol were only tentatively quantified, since they were below LOQ even for the amino acids which exhibited the highest sensitivity (LEU, ILE, PRO, PHE, TYR and TRP; Table 2). Despite the potential for high experimental errors in tentatively quantifying such low amino acid amounts, the results obtained were still useful as screening data in predicting amino acid turnover for a given developmental phase.

4. Conclusions

The determination of 15 essential amino acids in embryo culture media by derivatization and consequent analysis by GC using the presented procedure provided a straightforward and high-throughput means in analyzing amino acid composition of embryo culture media. The main requirement of the procedure was the absence of contamination in the chemicals and on the labware used. Sample reconcentration overcame the problems associated with water present in the sample solution and also provided extended method sensitivity. The procedure was also useful as a screening tool in the case when the amino acid content was below LOQ. The sensitivity could be even further improved if the source of chemical noise in the sample would be elucidated and prevented.

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