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FAST GC-MS METHOD FOR QUANTIFICATION OF GAMMA-BUTYROLACTONE IN BIOLOGICAL MATRICES

ANIKÓ PÉTER[a], TÍMEA DERGEZ[a], IBOLYA KISS[b], FERENC KILÁR[a,b]

ABSTRACT. A sensitive and specific gas chromatography-mass spectrometry analysis using selective ion monitoring for the quantification of γ-butyrolactone in biological matrices was developed. The method includes a simple liquid-liquid extraction and no derivatization procedure before the GC-MS analysis. An internal standard, α-methylene-γ-butyrolactone was applied for the quantitative determination. The method was linear between 0.34 µg/ml (LOD) and 500 µg/ml. The limit of quantification (LOQ) was obtained to be 0.798 µg/ml using 100 µl blood, urine or plasma.

Keywords: *gamma-butyrolactone, gamma-hydroxybutyric acid, GC-MS, blood, urine, plasma*

INTRODUCTION

Gamma-butyrolactone (γ-butyrolactone, GBL) is the chemical precursor of gamma-hydroxybutiric acid (γ-hydroxybutiric acid, GHB). Both are components of the normal mammalian metabolism, as endogenous constituents of the mammalian brain and they have been hypothesized to have a role as neurotransmitters [1]. Gamma-hydroxybutiric acid has been gaining popularity amongst club-goers as a recreational drug [2] . It is a component of “date rape drugs”, due to its euphoric effects [3] and ability to reduce inhibitions [4] or as doping agent (enhancer of muscle growth) [5]. Gamma-butyrolactone, on the other hand, has a hypothetical effect in the fungal metabolism, in filamentous fungi as a signalling molecule [6]. GBL has therapeutic importance, because of its pharmacokinetic and anti-angiogenic activity. Several quantitative analytical methods have been developed for measuring GHB and/or GBL in biological matrices [7-10]. Most of the papers report methods with gas chromatography-mass spectrometry, high performance liquid chromatography

[a] *Institute of Bioanalysis, University of Pécs, Faculty of Medicine, University of Pécs, Szigeti út. 12, H-7624 Pécs, Hungary, ferenc.kilar@aok.pte.hu*
[b] *Department of Analytical and Environmental Chemistry, Faculty of Science, University of Pécs, Ifjúság útja 6., H-7624 Pécs, Hungary*

or capillary electrophoresis procedures. The physiological level of gamma-hydroxybutiric acid is approximately 2 µg/ml in blood, and the normal endogenous concentration in urine is typically less than 10 µg/ml [11, 12]. Studies on the serum level of γ-butyrolactone, however, could not demonstrate its normal presence in significant amount, presumably due to its rapid conversion to γ-butyrolactone by γ-lactonase [13]. The determination of GHB was possible in the form of GBL after its acidic conversion [14]. The expected concentration of GBL in culture media of fungi is between 50-300 µg/ml [15, 16].

The aim of this study was to develop a rapid and simple method, for the quantitative determination of γ-butyrolactone in culture media of fungi, which will be applicable for the investigation of GBL in human blood and urine, as well. No method has previously been published for the determination of gamma-butyrolactone in culture medium.

RESULTS AND DISCUSSION

The gas-chromatography-mass-spectrometry analysis of γ-butyrolactone extracted by organic solvents from different biological matrices was studied. For the quantification of GBL an internal standard, α-methylene-γ-butyrolactone (M-GBL) was applied. Five solvents, chloroform, cyclohexane, dichloromethane, ethyl-acetate and methyl *tert*-butyl ether were examined in the extraction procedure. Care was taken to find the most suitable conditions for concentrating the extracts. A typical chromatogram of the two components after an extraction procedure with methyl *tert*-butyl ether (MTBE) using single ion monitoring is presented in Figure 1. The base peak of GBL at 42 *m*/*z*, and the base peak of M-GBL at 68 *m/z* can be clearly differentiated in the chromatogram, and besides these a fragment of GBL at 68 *m/z* can also be detected. The suggested fragmentation structure for the 42 *m/z* fragment is $[O=C=CH_2]^{+\cdot}$, while the M-GBL looses one carbon, one oxygen, and two hydrogens to form the 68 *m/z* fragment, it means most likely the elimination of $CH_2=O$ neutral molecule.

The quantification of γ-butyrolactone was obtained by using a calibration. The calibration curve for the standard was linear in the 0.34 - 500 µg/ml concentration range. Each correlation coefficient for three independent calibrations was R=0.999. The recovery experiments were carried out with a 500 µg/ml GBL concentration in blood, urine, or fungal culture. Cyclohexane and ethyl-acetate provided a recovery of γ-butyrolactone less than 20 % and 45 %, respectively. Although, the extraction with dichloromethane and chloroform resulted in a recovery higher than 90 %, due to the background noise, the LOD was higher than 200 µg/ml. The best recovery (95 %) was obtained with extraction using MTBE. The limit of quantitation was found to be 0.798 µg/ml (RSD: 20 %), and the limit of detection was obtained to be 0.34 µg/ml (RSD: 33 %).

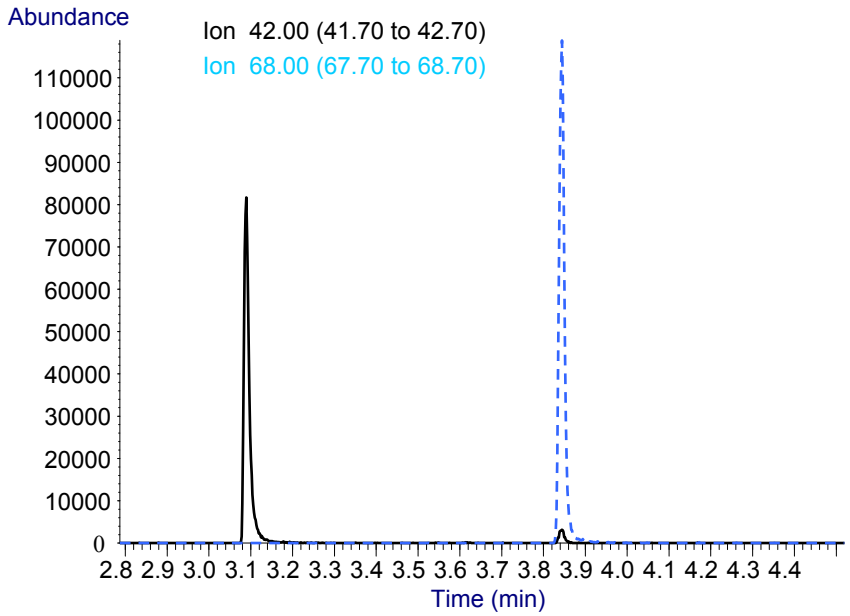

Figure 1. GC-MS chromatogram of a sample containing γ-butyrolactone and α-methylene-γ-butyrolactone. Single ion monitoring (SIM) detection of two fragment ions, 42 *m/z* (solid line) and 68 *m/z* (dashed line) was applied. The culture medium spiked with γ-butyrolactone (500 µg/ml) and α-methylene-γ-butyrolactone (500 µg/ml) was extracted with methyl *tert*-butyl ether. Other experimental conditions are given in the Experimental session.

The within-batch precision, using culture medium in the extraction, was between 1.1 to 3.9 %, the between-batch precision was between 1.7 to 7.3 % (experiments with three replicates). In the case of plasma, the within-batch precision was 4.9-8.1 %; in the case of blood cell suspension 10.1-13.4 %; and using urine the precision was between 0.8-5 %. The limit of detection was 0.34 µg/ml in culture medium and urine, and 0.5 µg/ml in blood. The limit of quantitation was 0.798 µg/ml in culture medium and urine, and 1.6 µg/ml in blood. The ratio of γ-butyrolactone present in the plasma and blood cells after centrifugation was *ca.* 45/55.

It was not possible to detect γ-butyrolactone in blank plasma or in blood cell suspension, which is in accordance to previous results, showing that the concentration of GBL in blank plasma is about 0.1 µg/ml [17].

The most efficient extraction was obtained with methyl *tert*-butyl ether (a low-boiling point solvent), but valid result could be obtained only after satisfactory sample preparation and within 40 min after sampling. The pretreatment procedure has been investigated with different solvents mainly with chloroform [7], but solid-phase extraction methods were also developed for the extraction of GBL [12]. Some publications applies salting-out approach [18], but those techniques are more complicated compared to the one described here.

CONCLUSIONS

The analytical gas-chromatography-mass-spectrometry method developed in this study is sensitive, accurate and precise for the determination of γ-butyrolactone, which participates in signal processes of fungi [15], but possibly occurent in human blood and urine. Only a simple pretreatment of the sample is necessary. The method provides a low LOD for the determination, although, applying special analytical conditions and instrumentation results with lower LOD values can be found in the literature [19]. In the case of following drug administration in human material a cautious interpretation of the data is necessary to avoid false positive results, therefore, it is mandatory to collect blood with EDTA, because the drug is cleared from the blood within 6 h [20].

EXPERIMENTAL SECTION

Reagents

Gamma-butyrolactone (GBL) and α-methylene-γ-butyrolactone (M-GBL) were purchased from Sigma-Aldrich (Darmstadt, Germany). Chloroform, cyclohexane, dichloromethane, ethyl-acetate and methyl *tert*-butyl ether (MTBE) were of GC grade. Stock solutions of GBL (10 mg/ml) were prepared in the organic solvents; M-GBL (10 mg/ml) was dissolved in MTBE. The Difco Potato Dextrose Broth (Difco Laboratories, Le Pont de Claix, France) was used (24 mg/ml) as culture medium. Drug-free human urine and blood were collected from healthy individuals. The blood samples were taken in EDTA tubes, and the biological samples were stored at 4°C.

Extraction

Extraction of GBL was made from aqueous solution, culture medium and from biological matrices containing different amounts of gamma-butyrolactone and 500 μg/ml α-methylene-gamma-butyrolactone, as internal standard. The concentration of GBL in the different solutions were as follows: aqueous solutions: 10 μg/ml or 50 μg/ml GBL; culture medium: 0, 0.4, 0.8, 1, 2.5, 5, 50, 100 and 500 μg/ml; blood or urine: 0, 0.5, 1, 2, 5, 10, 20 and 50 μg/ml.

The extraction procedure of the biological samples was preceded with a purification step, the samples in the culture medium were filtered through a Whatman Syringe Filter (0.45 µm), the urine was filtered with Millipore-Millex filter (0.45 µm), and the blood plasma was separated from the blood cells by centrifugation (3000 rpm, 30 min). The extraction was made applying 100 µl samples and 1 ml organic solvents (chloroform, cyclohexane, dichloromethane, ethyl-acetate or methyl *tert*-butyl ether). A suspension of the blood cells (100 µl) was also used for extraction. After vortexing (1 min. two times) and centrifugation (3000 rpm, 10 min) the organic part was collected and evaporated to *ca.* 100 µl final volume.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) analyses were performed on an Agilent 6890N GC equipped with a 5975 Inert Mass Selective detector (Agilent Technologies, Waldbronn, Germany). A HP-1 column (25 m x 0.2 mm I.D., 0.33 µm film thickness of polydimethylsiloxane) (Agilent Technologies, Waldbronn, Germany) was used for the separations applying helium as carrier gas at a flow rate of 1.5 ml/min. An HP 7683B automatic sampler was used for the injection. Split injection (20:1) was used with the valve closed for 2.7 min, and 1 µl samples being injected. The operating conditions for the analyses were: inlet temperature 250°C; the detector temperature 300°C; initial oven temperature was 50°C with a hold time of 0.6 minute and with a temperature ramp of 15°C min^{-1} up to 300°C. The mass spectrometer was operated in the selective ion monitoring (SIM) mode, monitoring GBL and M-GBL (the internal standard) by the 42 *m/z* and 68 *m/z* major fragment ions, respectively.

Method validation

The extraction was tested with γ-butyrolactone spiked distilled water (100 µg/ml). A calibration curve was constructed by preparing solutions in MTBE containing 0.5, 2.5, 5, 50, 100, 500 µg/ml GBL. The calibration curves were constructed in the case of the culture media by using 0, 0.4, 0.8, 1, 2.5, 5, 50, 100 and 500 µg/ml final γ-butyrolactone concentrations, and in the cases of blood and urine by using 0, 0.5, 1, 2, 5, 10, 20, 50 µg/ml final GBL concentrations.

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