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Journal of Chromatography B, 000 (2001) 000–000

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Sensitive and simple determination of mannitol in human brain tissues by gas chromatography–mass spectrometry

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Abstract

A simple, reliable and sensitive gas chromatographic–mass spectrometric method was devised to determine the level of mannitol in various human brain tissues obtained at autopsy. Mannitol was extracted with 10% trichloroacetic acid solution which effectively precipitated brain tissues. The supernatant was washed with *tert*.-butyl methyl ether to remove other organic compounds and to neutralize the aqueous solution. Mannitol was then derivatized with 1-butaneboronic acid and subjected to GC–MS. Erythritol was used as an internal standard. For quantitation, selected ion monitoring with *m/z* 127 and 253 for mannitol and *m/z* 127 for internal standard were used. Calibration curves were linear in concentration range from 0.2 to 20 μg/0.1 g and correlation coefficients exceeded 0.99. The lower detection limit of mannitol in distilled water was 1 ng/0.1 g. Mannitol was detected in control brain tissues, as a biological compound, at a level of 50 ng/0.1 g. The precision of this method was examined with use of two different concentrations, 2 and 20 μg/0.1 g, and the relative standard deviation ranged from 0.8 to 8.3%. We used this method to determine mannitol in brain tissues from an autopsied individual who had been clinically diagnosed as being brain dead. Cardiac arrest occurred 4 days later.

Keywords: Mannitol

1. Introduction

Mannitol is a sugar alcohol which is clinically used as a diuretic agent [1]. It is also used as a hyperosmolar solution that reduces brain water content by establishing osmotic gradient in the treatment of elevated intracranial pressure (ICP), as a result of brain trauma [2,3]. At forensic autopsies, we often experience the cases where the patients had suffered from these trauma caused by traffic accidents, fighting or labor accidents. In these cases, many patients fall into brain death before cardiac death [4]. Therefore, forensic diagnosis has to be made as to when and how the patients actually became brain dead.

We have reported the specific distribution pattern of drugs in the brain due to the cessation of cerebral blood flow by analyzing administered phenobarbital [5], diazepam [6], or caffeine [7]. The possibility of diagnosing brain death by analyzing these drugs seemed feasible. However, these drugs are not always involved in case of brain injured patients. On the other hand, mannitol is commonly administered in the state of elevated ICP. Therefore, we attempted to analyze this drug in brain tissues.

In studies on humans, mannitol has been analyzed using biological fluids [8–13], and studies on the determination of mannitol in human solid tissues have apparently not have been reported. Such being

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00145-1

the case, we designed a sensitive and simple method to determine level of mannitol in autopsied brain tissues, using gas chromatography–mass spectrometry (GC–MS).

2. Experimental

2.1. Reagents

Mannitol and erythritol were purchased from Wako (Osaka, Japan) and 1-butaneboronic acid was from Aldrich (Milwaukee, WI, USA). Trichloroacetic acid was purchased from Ishizu (Osaka, Japan) and *tert*.-butyl methyl ether, purchased from Tokyo Kasei Kogyo (Tokyo, Japan) was distilled prior to use. All chemicals used in this study were of analytical grade.

2.2. Biological samples

Human brain tissue samples were obtained at the time of autopsy and stored at −20°C until analysis. Drug-free whole blood and tissues were used as control samples.

2.3. Standard solution of mannitol and internal standard (I.S.)

Mannitol (100 mg) was dissolved in distilled water to give a concentration of 10 μg/μl and this solution was further diluted to obtain concentrations of 1000, 100, 10, 1 ng/μl. The standard solution of I.S. (erythritol) was prepared by dissolving 10 mg of erythritol in 10 ml of methanol.

2.4. Extraction procedure

The method of extracting mannitol from human brain tissues was as follows: a 0.1-g amount of tissue was weighed and homogenized in 2 ml of 10% trichloroacetic acid and 10 μl of I.S. solution (10 μg of erythritol in methanol), and the preparation was centrifuged at 7000 *g* for 15 min. The supernatant was transferred to another 10-ml centrifuge tube and 2 ml of *tert*.-butyl methyl ether was added, then the preparation was shaken briefly and centrifuged at 2000 *g* for 5 min, this step was repeated once more. The organic layer was removed and 200 μl of the aqueous layer was placed in a microtube and evaporated to dryness under a stream of nitrogen at 70°C. The residue was dissolved in 10 μl of 1-butaneboronic acid solution (5 mg/ml in ethanol) and sonicated for 10 min. A 1-μl aliquot of the solution was injected onto the GC–MS system. Duplicated analyses were carried out for each sample.

2.5. Preparation of calibration curves

Calibration curves at concentrations of 0.2–20 μg/0.1 g were prepared by adding mannitol to the brain tissues homogenized with 10% trichloroacetic acid solution. These samples were extracted in the same manner as described above. Calibration curves were obtained by plotting peak area ratios of mannitol to I.S. versus the amounts of mannitol.

2.6. GC–MS conditions

The apparatus used was a Hewlett-Packard 5971 mass-selective detector with a Hewlett-Packard 5890 II gas chromatograph. The HP-5 fused-silica capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness) was coated with 5% phenyl methyl silicone. Splitless injection mode was selected with a valve off-time 2 min.

The GC operating conditions were programmed at a rate of 30°C/min from 100 to 200°C, the program was then slowed down to 20°C/min until 300°C was reached and this temperature was maintained for 1 min. Injection port and transfer line temperatures were both 280°C. Helium was used as a carrier gas with a column head pressure of 50 kPa. The ionization energy was 70 eV. For quantitation, the ions at *m*/*z* 127 and 253 for mannitol and *m*/*z* 127 for I.S. were selected.

3. Results and discussion

3.1. Extraction procedure

Most drugs in clinical use are hydrophobic and can be extracted using organic solvents. Comparatively speaking, mannitol dissolves poorly in organic

solvents, hence extraction is difficult using ordinary techniques. Mannitol in biological fluid has been analyzed after precipitation of protein using ethanol, acetone, iced sulfosalitilic acid, zinc sulfate and barium hydroxide [8,9,11,14–17]. As these agents did not precipitate sufficient protein from brain tissues, we used 10% trichloroacetic acid solution, and protein precipitation was complete. Interfering organic compounds and trichloroacetic acid were effectively removed by washing the supernatant with *tert*.-butyl methyl ether and clean extracts were obtained. A solid-phase extraction procedure was also tested, but as it was much more time consuming, our liquid–liquid extraction procedure is more economical and readily facilitated.

3.2. Derivatization procedure

Mannitol is a carbohydrate with six hydroxy groups and with no UV absorbing functional groups. To detect intact mannitol, pulsed amperometric detection (PAD) has been used for analysis by high-performance liquid chromatography (HPLC) [10,17]. For UV detection of mannitol by HPLC, a derivatizing procedure such as *p*-nitrobenzoylation is essential [18,19]. For analysis of mannitol by GC–MS,

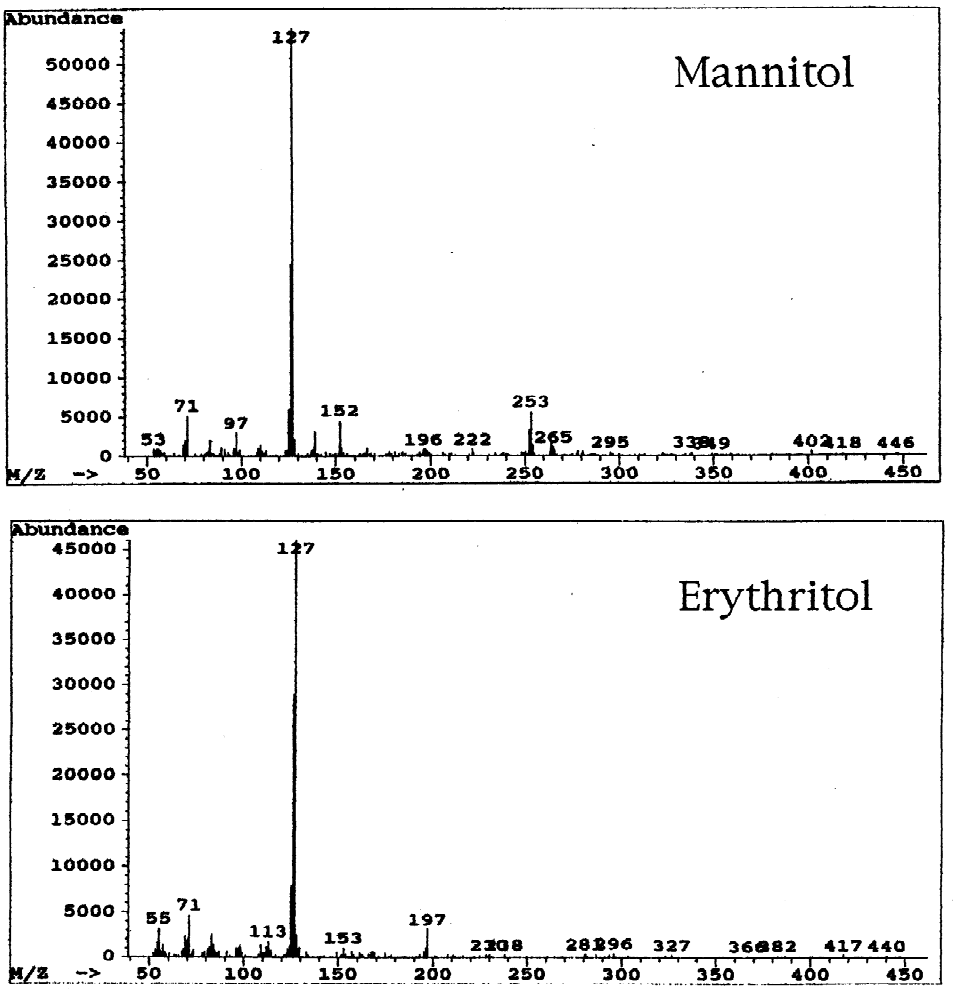

Fig. 1. EI mass spectra of mannitol and erythritol.

other derivatization procedures such as trimethylsilylation (TMS) [20], acetylation [21] and *n*-butyldiboronation [22] have been used. As mannitol has bifunctional hydroxyl groups, organic boronic acids are most useful reagents to use for derivatization, as compared with TMS or acetate. An *n*-butyldiboronate derivative of mannitol gave a better separation from other polyols and only 10 min at room temperature was required for derivatization [23]. Therefore, we used 1-butaneboronic acid and GC–MS to determine mannitol levels in brain tissues.

3.3. GC–MS determination of mannitol in human brain tissues

Electron impact (EI) mass spectra of mannitol and erythritol are shown in Fig. 1. Major fragment ions

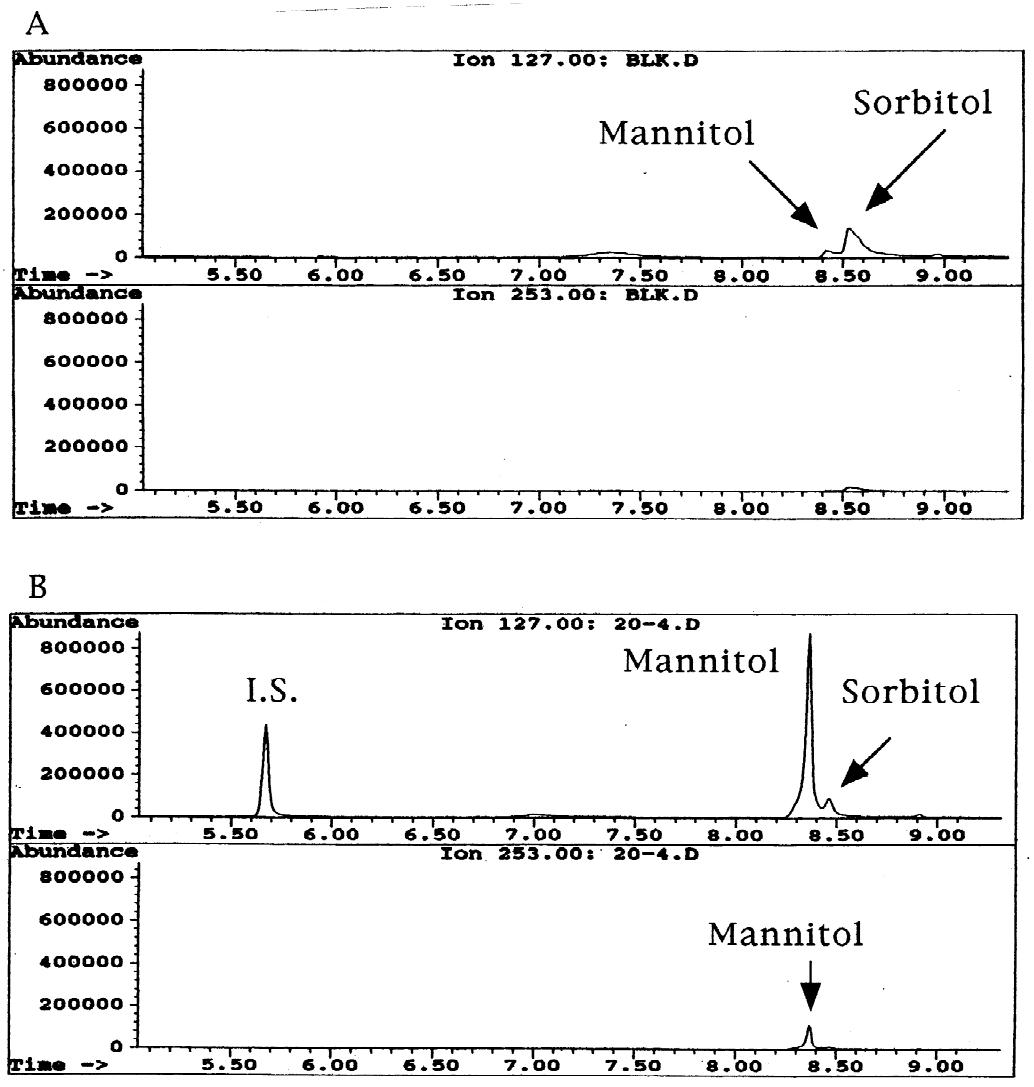

Fig. 2. Selected ion monitoring chromatograms of extracts from 0.1 g of the human brain tissues. (A) Control brain. (B) Control brain containing 20 μg of mannitol and 10 μg of I.S.

were observed at m/z 127, 152 and 253 for mannitol, and m/z 127 and 197 for erythritol. Each ion was examined and ions m/z 127 and 253 for mannitol and m/z 127 for erythritol were selected for quantitation. Fig. 2 shows selected ion monitoring chromatograms of the extracts from 0.1 g of human control brain (A) and those from 0.1 g of human brain containing 20 μg of mannitol and 10 μg of I.S. On the chromatogram for control brain tissues (Fig. 2A), about 50 ng/0.1 g of mannitol, as a biological compound, was detected with the peak of sorbitol. Although slight overlap of sorbitol with mannitol was observed, each ion abundance ratio (M127/E127, M253/E127) gave good linearity with correlation coefficients over 0.99 in concentration ranges from 0.2 to 20 μg/0.1 g. As the control human brain tissues contained mannitol about 50 ng/0.1 g, we determined the lower limit of detection by adding mannitol to distilled water, this was 1 ng/0.1 g with a signal-to-noise ratio of 3:1, and we confirmed this method to be useful for our purpose. The recovery of mannitol in tissues was determined by adding 2 or 20 μg of mannitol to 0.1 g of control human brain tissue and submitting the same analytical procedures described above. The absolute recovery was calculated by comparing the peak area of mannitol in tissue extracts with that of mannitol in standard solution. The calculated recoveries ranged from 96.5 to 123.4% in both concentrations. The high recovery of mannitol may be derived from a different matrix between brain tissue and water, where mannitol in standard solution would be more retentive to the column than in brain tissue. The precision of this method for examining concentrations of 2 and 20 μg/0.1 g in human brain tissues is summarized in Table 1 (n=5). The relative standard deviations ranged from 0.8 to 8.3% and good accuracy and precision were obtained.

Table 1
Within- and between-day variations for mannitol

Mannitol/I.S. (m/z)	Mannitol concentration (μg/0.1 g)	
	2	20
Within-day RSD (%) (n=5)		
127/127	1.99	0.84
253/127	1.92	5.65
Between-day RSD (%) (n=5)		
127/127	8.34	4.72
253/127	4.76	5.53

4. Practical application

An 18-year-old Japanese man was found lying in a railway station yard. Light, corneal and brain stem reflection were nil, and spontaneous respirations were absent when he was first brought to a hospital. CT scans showed bilateral swelling of the brain. 20% mannitol in water was intravenously administered with the total volume of 1000 ml on the first hospital day. Cardiac arrest occurred 4 days later. At autopsy, we determined the concentrations of mannitol in his brain using the method described herein. Mannitol in different concentrations was clearly present in all brain tissues as shown in Table 2, but levels in the whole blood were much lower. This variation in mannitol distribution is pertinent for diagnosing the time and progress of brain death. The official cause of death was documented as cerebral contusion following a railroad accident.

5. Conclusion

A sensitive and simple method was devised to determine levels of mannitol in autopsied human

Table 2
Concentrations of mannitol in autopsied human tissues

Sample	Concentration (μg/0.1 g)	
	Left area	Right area
Occipital lobe	1.5	3.4
Frontal lobe	1.3	3.1
Temporal lobe	2.4	3.2
Parietal lobe	1.2	2.3
Cerebellum	3.3	
Mesencephalon	2.8	
Pons	3.1	
Medulla oblongata	3.1	
Femoral blood	0.7	
Heart blood	0.9	

brain tissues, using GC–MS. The forensic assessment of distribution of mannitol in human brain tissues was thus made feasible.

Acknowledgements

We thank M. Ohara for language assistance.

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