Journal of Chromatography, 617 (1993) 140–146
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6893

Short Communication

Determination of phloroglucinol in human plasma by gas chromatography–mass spectrometry

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(First received March 3rd, 1993; revised manuscript received April 22nd, 1993)

ABSTRACT

A specific and sensitive method has been developed for the determination of phloroglucinol in plasma; it involves an optimized procedure for blood sampling designed to minimize the *in vitro* oxidation of the molecule, and gas chromatography–mass spectrometry after silylation of the compound. The method allowed a reliable determination of phloroglucinol in plasma. The precision and accuracy of the assay, reported as coefficients of variation, were below 15%. Using a plasma sample of 0.25 ml, the limit of quantitation was 5 ng/ml with a precision of 17.4%, which is sensitive enough for pharmacokinetic studies. Stability studies under different conditions revealed that ascorbic acid limits the degradation of phloroglucinol in plasma during storage at freezer temperatures.

INTRODUCTION

Phloroglucinol (benzene-1,3,5-triol) is used as a smooth muscle relaxant. It has no anticholinergic potency and appears to be less toxic than most other antispasmodic agents. Previously reported methods for the determination of phloroglucinol are scanty, concerned only urine samples and used colorimetry [1] or were carried out on plants [2,3]. Pesez and Bartos [4] have shown that, although this compound does not fluoresce naturally, it can be converted into a fluorescent derivative by condensation with ethyl iodoacetate to form a coumarin derivative. Thus, a high-performance liquid chromatographic (HPLC) method combined with fluorimetric detection might be used to determine phloroglucinol. However, we could find no published method for the determination of phloroglucinol in plasma. Thus, the pharmacokinetics of this compound are unknown, probably owing to the lack of an adequately sensitive and specific method and the instability of phenolic compounds, which are known to be very sensitive towards oxygen and light [2].

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0378-4347/93/$06.00

This paper describes a specific and sensitive gas chromatographic–mass spectrometric (GC–MS) method, which appears to be suitable for pharmacokinetic studies of phloroglucinol in humans. Major efforts have been made to develop an optimized procedure for blood sample collection, storage and thawing, based on practical solutions described by Chamberlain [5]. Indeed, phloroglucinol appears to be unstable in plasma, even stored at −20°C or at −80°C, unless ascorbic acid is added as an antioxidant before freezing.

EXPERIMENTAL

Chemicals and reagents

All reagents and solvents were of analytical-reagent grade. The phosphate buffer solution (pH 7.0) was obtained from Merck (Darmstadt, Germany), as were the extraction solvent, ethyl acetate, and the stabilizing agent, ascorbic acid. The derivatization reagent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL, USA). Phloroglucinol was a gift of Fabre Labs. (Castres, France) and resorcinol, used as the internal standard, was supplied by Merck. Stock solutions at 1 mg/ml and working solutions were prepared daily before analysis in distilled water.

Gas chromatography–mass spectrometry

The GC–MS system consisted of an HP (Hewlett Packard, Avondale, PA, USA) 5890 Series II gas chromatograph connected to an HP 5970 A mass-selective detector. GC analysis was performed on a WCOAT CP-Sil 19 fused-silica capillary column (20 m × 0.22 mm I.D., 0.2 μm film thickness, Chrompack, Middelburg, Netherlands), protected by a deactivated fused-silica precolumn (1 m × 0.22 mm I.D., SGE, Villeneuve-Saint-Georges, France). Helium, at 0.7 bar (*ca.* 520 Torr), was used as the carrier gas. The oven temperature was initially set at 60°C for 0.5 min, then programmed at 35°C/min to 270°C. The injector and the transfer line temperatures were set at 250°C and 280°C, respectively.

Extraction and derivatization procedures

Plasma (0.25 ml) was spiked with resorcinol as the internal standard (50 μl at 4 μg/ml), diluted with 0.75 ml of the buffer solution (pH 7.0), and extracted with 4.5 ml of ethyl acetate in a reciprocating shaker for 10 min. After centrifugation for 10 min at 1600 *g*, the organic layer was transferred to 5-ml polypropylene tubes and concentrated at room temperature to a volume of *ca.* 0.25 ml using a Speed Vac concentrator (Speed Vac SC100, Savant, France). The organic phase was then quantitatively transferred into 1.5-ml Eppendorf tubes and evaporated to dryness using the Speed Vac concentrator.

Derivatization was carried out by adding 50 μl of BSTFA to the evaporation residue. The reaction mixture was stirred on a vortex mixer for 30 s and allowed to stand for 15 min at room temperature. A 1-μl volume was injected into the GC–MS system using an autosampler.

Plasma samples

Collection and storage. Blood samples (5 ml) for the pharmacokinetic study were collected in heparin–lithium tubes, then immediately centrifuged for 10 min at 1600 *g* and 10°C. Plasma was separated in 0.25-ml fractions under filtered light. An aqueous solution of ascorbic acid (0.2 ml at 0.2 g/l) was added to each fraction to prevent oxidation, as reported for apomorphine [6]. Fractions were then kept frozen at −20°C until analysis in 0.5-ml Eppendorf tubes, in order to minimize the apple-jack effect pointed out by Scales [7].

Thawing. Plasma samples were thawed at room temperature. At the end of thawing, which was rapid owing to the small volume of the aliquot, both the sample and 2 × 0.375 ml of the buffer solution (used to rinse the freezing tubes) were transferred to 10-ml polypropylene extraction tubes containing the internal standard (resorcinol, 50 μl at 4 μg/ml).

Calibration curves. To establish the calibration curves, fresh plasma samples (0.25 ml) were introduced into 10-ml polypropylene tubes containing an aqueous solution of ascorbic acid (0.2 ml at 0.2 g/l) and the internal standard, then

spiked with various amounts of phloroglucinol. The resulting concentrations were 0, 10, 20, 40, 100, 200, 400, 600, 800, 1000 and 1200 ng/ml.

Stability study

To control the stability of phloroglucinol in frozen plasma samples, a three-step study was carried out at different concentrations covering the range of expected values. Plasma samples were analysed after storage for two months. Analyses were performed at various time intervals (0, 1, 2, 7, 10, 14, 21, 45 and 60 days) under different conditions: (i) at −20°C, without a stabilizing agent; (ii) at −80°C, without a stabilizing agent; and (iii) at −20°C with ascorbic acid.

Quality controls

Specimens were prepared in 0.5-ml Eppendorf tubes according to the optimized procedure, and were spiked at levels covering a range from blank to the highest anticipated concentration by a technician who did not participate in the study. The quality control samples (n = 27) were stored frozen at −20°C until analysis. They were analysed blind and at the same time as the plasma samples of the stability study.

Preliminary pharmacokinetic assay

A healthy volunteer, after giving full informed consent, received phloroglucinol (160 mg) as a single oral dose. Venous blood was withdrawn before administration, then at 3, 10, 20, 30 and 45 min and 1, 1.5, 2, 3, 4, 5 and 6 h following administration of the drug.

RESULTS AND DISCUSSION

Selected-ion monitoring

Mass spectra of silylated derivatives of phloroglucinol and resorcinol are shown in Fig. 1. Quantitative analysis was carried out with the molecular ions, *i.e.* the ion at m/z 342 for phloroglucinol and the ion at m/z 254 for resorcinol. Typical chromatograms of plasma extracts from a healthy volunteer before and after oral administration of 160 mg of phloroglucinol are shown in Fig. 2. Quantitation was carried out by following the m/z 342 (phloroglucinol) / m/z 254 (resorcinol) ratio.

Validation

The extraction efficiency with ethyl acetate was determined at three concentrations of phloroglucinol, *i.e.* 200, 600 and 1200 ng/ml (three replicates for each level) and at 800 ng/ml for resorcinol (six replicates). Extraction recoveries were above 85% for both phloroglucinol and resorcinol, and were 96.6 ± 6.3%, 85.0 ± 4.2% and 88.9 ± 6.1% at 200, 600 and 1200 ng/ml phloroglucinol, respectively, and 89.2 ± 5.8% at 800 ng/ml resorcinol.

Calibration graphs were constructed for the ranges 0–1200 ng/ml (eleven points) by plotting the peak-area ratio of the ion at m/z 342 (phloroglucinol) and the ion at m/z 254 (resorcinol) *versus* the concentration of phloroglucinol. Linear regression analysis showed that the linearity was satisfactory. The equation of the mean plot (n = 9) when ascorbic acid was used as stabilizer was $y = 0.00381x + 0.0822$ (r = 0.998); the slopes of calibration graphs were reproducible: 0.00399 ± 0.000535 (C.V. = 13.4%); and the mean correlation coefficient was 0.998 ± 0.001.

To quantitate low concentrations of phloroglucinol in plasma, it appears to be more accurate to establish a separate curve for the low concentration range, *i.e.* 0–200 ng/ml. The linear regression of the mean plot (n = 9) for the low concentration range was $y = 0.00434x + 0.0361$ (r = 0.999); the slopes were reproducible (0.00453 ± 0.00082); and the mean correlation coefficient was 0.999 (± 0.001).

The precision and accuracy were determined by analysing ten replicate plasma samples at 5, 100, 600 and 1200 ng/ml. The calculated concentrations of phloroglucinol (mean ± S.D.) are reported in Table I.

The background signal, as shown in Fig. 2a, although close to zero, appears to vary between assays. In order to determine an accurate limit of quantitation, the statistical approach proposed by Girault *et al.* [8] was used. The mean calculated value ± S.D. for ten blank plasma samples was 0.82 ± 0.22 ng/ml. The theoretical limit of

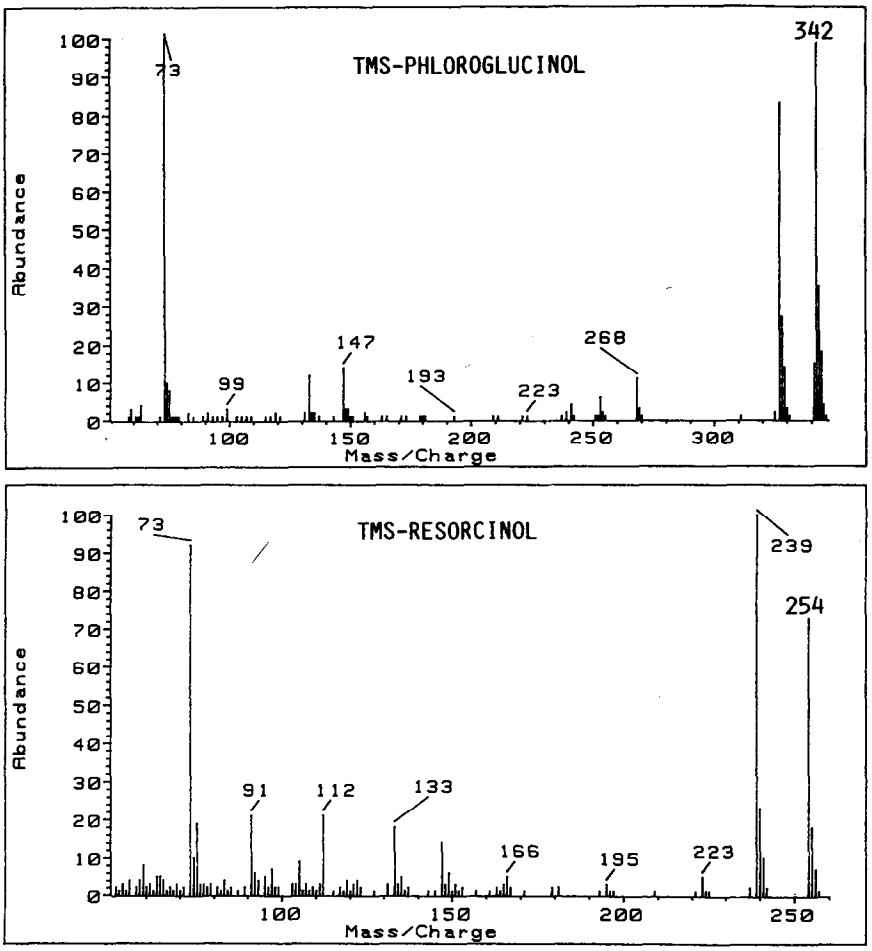

Fig. 1. Electron impact mass spectra of silylated derivatives of phloroglucinol and resorcinol.

TABLE I

PRECISION AND ACCURACY OF THE PHLOROGLUCINOL ASSAY

Concentration added (ng/ml)	Concentration found (mean ± S.D., n = 10) (ng/ml)	Precision[a] (%)	Accuracy[b] (%)
5[c]	4.6 ± 0.8	17.4	−8.0
100	97.1 ± 14.0	14.4	−2.9
600	570.5 ± 64.9	11.4	−4.9
1200	1181.1 ± 64.6	5.5	−1.6

[a] Coefficient of variation = standard deviation/mean × 100.
[b] Percentage deviation = (found − added)/added × 100.
[c] Limit of quantitation.

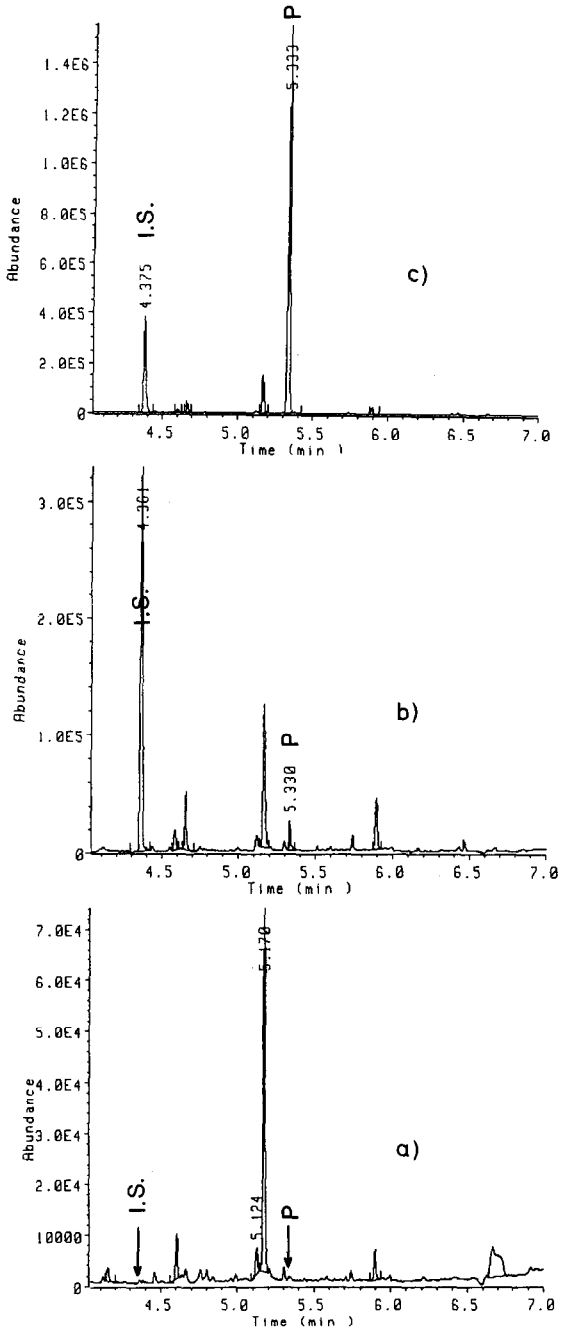

Fig. 2. Selected-ion monitoring (SIM) signals of a blank plasma sample (a) and plasma samples spiked with resorcinol as internal standard (I.S.) and with phloroglucinol (P) at the limit of quantitation (5 ng/ml) (b) and at 800 ng/ml (c).

detection, defined as the concentration yielding a significantly higher signal ($p < 0.05$) than blank specimens was 1.95 ng/ml. However, the precision of the assay at this concentration was insufficient. Thus, the lowest concentration that was significantly different from blank samples ($p < 0.001$) and could be quantified with sufficient precision (C.V. = 17.9%, $n = 10$) was 5 ng/ml. The SIM signal from a plasma sample spiked at this concentration is shown in Fig. 2b.

Stability

Results of stability study, expressed as mean calculated concentrations (±S.D.), are reported in Table II. Degradation of phloroglucinol occurred in plasma samples as early as 24 h following freezing at −20°C or at −80°C, unless ascorbic was added before freezing. Nevertheless, even under these conditions, the stability of phloroglucinol in plasma may be considered as quite reliable for only two weeks, and cannot exceed 30 days. A decrease of theoretical values (higher than 20%) was observed after 45 days at −20°C, even in presence of ascorbic acid. The results of analysis of quality control samples are in good agreement with these results. They showed that calculated values are correlated with the theoretical values with a mean deviation of *ca.* −6.4% for the quality controls determined during the first month ($n = 21$), and the mean error increases to 30% after this.

This has to be taken into account when planning a pharmacokinetic study of phloroglucinol.

Pharmacokinetic assay

The applicability of the method to pharmacokinetic studies is shown in Fig. 3. The phloroglucinol concentration was determined within two days following the administration and sampling. The peak drug concentration (C_{max} = 677 ng/ml) after administration of 160 mg of phloroglucinol was reached after 20 min. The half-life was *ca.* 1.5 h, and after 6 h the concentration had fallen to 9 ng/ml.

CONCLUSIONS

The method presented here proved to be specific and sensitive. With only 0.25 ml of plasma, the limit of quantitation was 5 ng/ml. This limit

TABLE II

SAMPLE STABILITY DURING STORAGE

Storage		Concentration (ng/ml)		C.V. (%)	Mean error (%)
Conditions	Days	Spiked	Found (mean ± S.D., n = 3)		
−20°C	0	20	19.4 ± 1.5	7.7	−3.0
	1		16.4 ± 3.5	21.4	−18.0
	2		11.0 ± 1.5	13.5	−45.0[a]
	10		9.4 ± 0.6	6.1	−53.0[a]
	0	200	181.8 ± 15.2	8.3	−18.2
	1		134.6 ± 12.9	9.6	−32.7[a]
	2		140.9 ± 9.9	7.0	−30.0[a]
	10		114.5 ± 17.5	15.3	−42.8[a]
	0	800	723.2 ± 79.1	10.9	−9.6
	1		554.4 ± 181.0	32.6	−30.7[a]
	2		517.0 ± 19.4	3.7	−35.4[a]
	10		448.0 ± 66.7	14.9	−44.0[a]
	0	1600	1358.2 ± 82.3	6.0	−15.1
	1		953.2 ± 100.3	10.5	−31.9[a]
	2		960.6 ± 35.8	3.7	−31.4[a]
	10		852.4 ± 52.0	6.1	−39.1[a]
−80°C	0	20	19.0 ± 0.8	4.3	−5.0
	1		12.1 ± 1.4	11.6	−39.5[a]
	2		14.0 ± 1.9	13.6	−30.0[a]
	0	800	648.0 ± 49.8	7.7	−19.0
	1		513.6 ± 38.7	7.5	−35.8[a]
	2		478.7 ± 79.3	16.6	−40.2[a]
	0	1600	1302.3 ± 31.9	2.4	−18.6
	1		1015.3 ± 69.5	6.8	−36.5[a]
	2		1060.7 ± 190.3	17.9	−33.7[a]
−20°C	0	20	18.0 ± 1.6	8.9	−10.0
+ ascorbic	1		18.3 ± 0.6	3.3	−8.5
acid	7		23.7 ± 2.5	10.5	+18.5
before	14		20.7 ± 0.9	4.3	+3.5
freezing)	28		22.0 ± 3.7	16.8	+10.0
	45		21.0 ± 1.9	9.0	+5.0
	0	100	99.2 ± 5.0	5.0	−0.8
	1		94.7 ± 1.5	1.6	−5.3
	7		96.2 ± 3.3	3.4	−3.8
	14		99.2[b]		−0.8
	28		86.7 ± 5.5	6.3	−13.3
	45		68.4 ± 1.8	2.6	−31.6[a]
	0	600	667.7 ± 32.0	4.8	+11.3
	1		546.0 ± 49.9	9.1	−9.0
	7		588.3 ± 44.6	7.6	−2.0
	14		600.7 ± 33.7	5.6	+0.1
	28		510.7 ± 3.1	0.6	−14.9
	45		435.5 ± 31.8	7.3	−27.4[a]
	0	1200	1301.0 ± 32.4	2.5	+8.4
	1		1351.0 ± 23.4	1.7	+12.6
	7		1203.0 ± 86.0	7.1	+0.2
	14		1150.0 ± 36.6	3.2	−4.2
	28		1038.0 ± 22.9	2.2	−13.5
	45		899.0 ± 39.1	4.3	−25.1[a]

[a] Mean error >20%. Unacceptable storage conditions.

[b] Sample with n = 1.

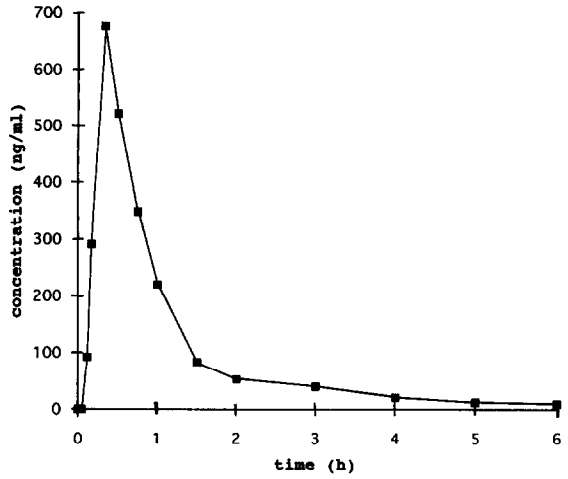

Fig. 3. Graph of plasma concentration *versus* time for phloroglucinol in a healthy volunteer receiving a single oral dose of 160 mg of phloroglucinol.

may be improved by using 1 ml of sample. Because of the rapid elimination of this drug, a high sensitivity is required for its pharmacokinetic study. Stability studies revealed the high instability of the drug in plasma and the necessity for the addition of ascorbic acid before freezing in order to slow down the degradation. These different problems may explain, in part, the lack of previously reported data concerning the pharmacokinetics of this drug.

Under the conditions described here, the stability of samples was satisfactory for two weeks. Nevertheless, plasma samples should not be stored frozen for more than one month before analysis.

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