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Short Communication

Determination of a new blood glucose-lowering agent in biological fluids by capillary gas chromatography using electron-capture detection

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ABSTRACT

A gas chromatographic method for the determination of 5-(4-chlorophenyl)-2-[(4-methylphenyl)sulphonyl]-4-pentynoic acid (I) in plasma (serum) and urine has been developed. After an extraction process, the cleaned-up organic extract was derivatized with diazomethane at ambient temperature. Results are evaluated from peak-height ratios with respect to the appropriate internal standard. The detection limit following extraction of a 1-ml plasma sample is about 20 ng/ml.

INTRODUCTION

Peripheral insulin resistance is thought to be one of the most important mechanisms in the pathophysiology of non-insulin-dependent diabetes mellitus (NIDDM) [1–3]. Therefore a compound which can reduce such a resistance may be of advantage in the treatment of NIDDM. During a comprehensive research programme a new class of compounds with an effect on peripheral insulin resistance was found. One of these compounds is 5-(4-chlorophenyl)-2-[(4-methylphenyl)sulphonyl]-4-pentynoic acid (I, Fig. 1). This is a blood glucose-lowering agent which is effective in diabetic animal models [4,5].

For pharmacokinetic studies in man and animals a simple, highly reliable and sensitive method for the determination of this compound in biological fluids was developed. This method uses an internal standard, 2-[(4-methylphenyl)sulphonyl]-5-(4-methylphenyl)-4-pentynoic acid (II, Fig. 1), liquid–liquid extraction, derivatisation and gas chromatography (GC) with an electron-capture detector.

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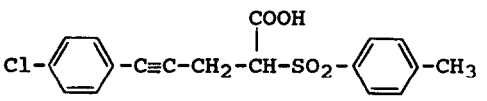
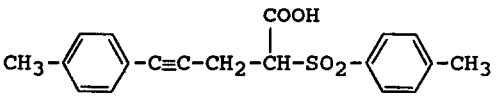


I



II

Fig. 1. Structures of I and II (the internal standard).

EXPERIMENTAL

Reagents

The reference standards (I and II) were synthesized in the author's laboratories [6,7]. Analytical-reagent grade reagents were used as supplied by the manufacturers. The following procedure was used for the preparation of diazomethane: to a cold mixture of 14 ml of 40% aqueous potassium hydroxide solution and 60 ml of diethyl ether, 6 g of 1-methyl-3-nitro-1-nitrosoquanidine were added slowly (temperature $< 5°C$). After standing for about 10 min the organic phase was transferred into a flask, diluted with 50 ml of diethyl ether and the flask sealed. The reagent was stored in a freezer.

Apparatus and gas chromatographic conditions

The GC system consisted of a Hewlett-Packard Model 5880 or 5890 gas chromatograph with a ^{63}Ni electron-capture detector, an HP 7672 A automatic sampler (Hewlett-Packard, Böblingen, Germany) and a Kipp & Zonen recorder (Solingen, Germany).

The measurements were carried out with a DB5 15 m × 0.32 mm I.D. fused-silica column (95% dimethyl, 5% diphenyl polysiloxane, film thickness 0.25 μm) from ict Handelsgesellschaft (Frankfurt, Germany). The operating conditions for routine analysis were: oven temperature program, 200°C for 1 min then 10°C/min to 280°C; injection port temperature, 300°C; detector temperature, 300°C; injection volume, 1 μl. The gas flow-rates were: carrier gas, 8–10 ml/min; hydrogen make-up gas, 22 ml/min (5880) and 50 ml/min (5890); 5% methane in argon, split 100 ml/min after 30 s. The total analysis time was about 14 min.

Procedure

Depending on the sample concentration, 10–100 μl of plasma, serum or urine were transferred into a 10-ml glass-stoppered centrifuge tube and diluted with 1 ml of drug-free control plasma (A) or 1 ml of deionized water (B). To each sample 50 μl of internal standard solution (20 μg/ml in acetonitrile) were added. After the

addition of 3 ml (A) or 4 ml (B) of diethyl ether and 25 μl of concentrated hydrochloric acid, the tubes were shaken in a tube rack on a reciprocating shaker for 10 min and centrifuged at 4000 *g*. The organic phase was transferred into a 10-ml conical centrifuge tube and evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 300 μl of ethyl acetate, transferred into a sample vial and evaporated to dryness at 50°C under a stream of nitrogen. For derivatisation the residue was taken up in 100 μl of diazomethane. After 30 min at room temperature the solution was evaporated to dryness and, depending on the sample concentration, the residue was taken up in 50–100 μl of acetonitrile. The tubes were then sealed and vortex-mixed. A 1-μl volume was injected into the GC system.

Calibration graphs were prepared by assaying plasma, serum or urine samples to which known amounts of I and II had been added. The peak-height ratios of I to II were plotted *versus* the amounts of I added.

For the experimental fit of the data points to the regression line three modes of regression were used: linear, power-fit and non-linear regression. The coefficients of correlation of the different regression equations were routinely calculated to evaluate the fit of the calibration data to the regression line (>0.99). The function with the best correlation was used for the calibration of the sample concentrations.

RESULTS

Selectivity

Drug-free control human plasma shows no significant peaks at the retention times of I or II (internal standard) when analysed by this method (see Fig. 2A). Fig. 2B shows the chromatogram of an extract of 1 ml of human plasma spiked with 25 μg/ml I and 10 μg/ml internal standard. Fig. 2C shows the chromatogram of an extract of 100 μl of a human volunteer plasma sample taken 30 min after a single dose of 200 mg of I.

The identity of I and II in the derivatized form as methyl esters was confirmed by gas chromatography–mass spectrometry (GC–MS).

Accuracy and precision

To determine between-run and within-run precision and accuracy a plasma pool was spiked with 50 and 5 μg/ml I. At six days six samples of each concentration were analysed giving a total of 36 samples. The mean and standard deviation calculated from the six measurements of each work-up represent the within-run accuracy and precision. The between-run accuracy and precision are indicated by the mean and standard deviation of the six within-run calculations.

Tables I and II show the results of these measurements.

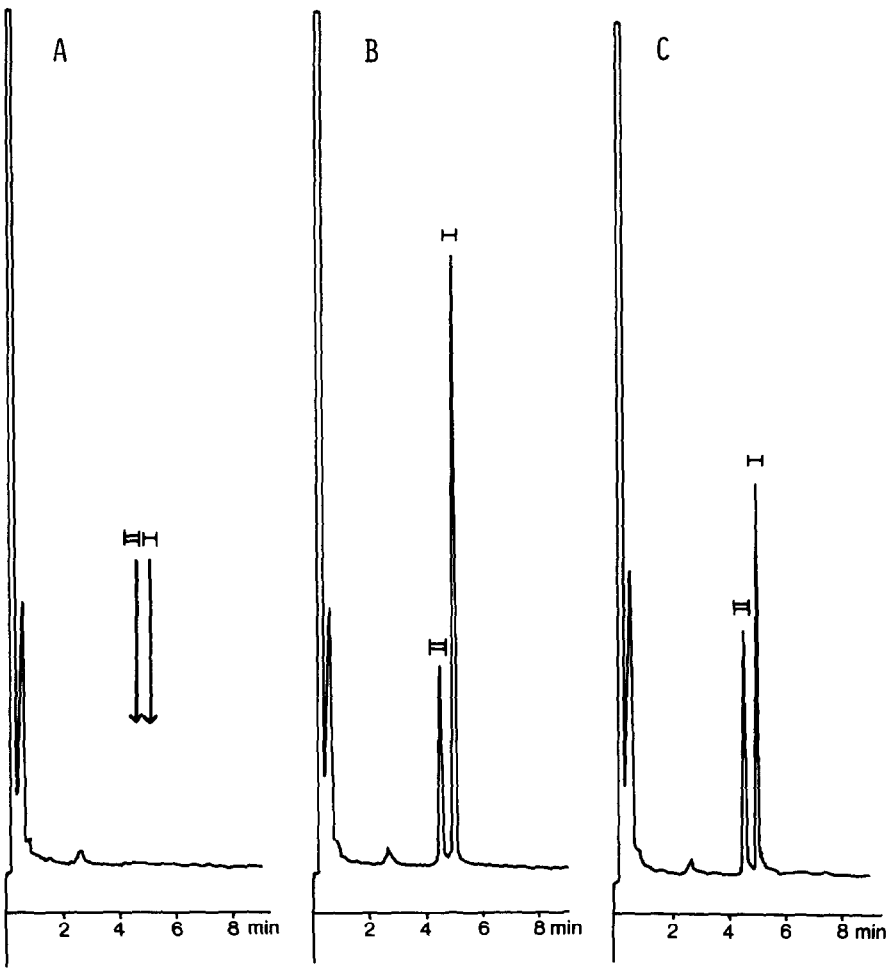


Fig. 2. (A) Chromatogram of a drug-free control human plasma sample. (B) Chromatogram of a human plasma sample spiked with 25 μg/ml I and 10 μg/ml II (internal standard). Sample volume, 100 μl, diluted with 1 ml of control plasma. (C) Chromatogram of an extract of a 100-μl human plasma sample (diluted with 1 ml of control plasma) of a volunteer taken 30 min after a single oral dose of 200 mg of I. The concentration of I was 13.2 μg/ml.

Recovery

The recovery of I and II was studied (A) in plasma samples and (B) in water-diluted plasma samples. The procedure used for this study is given in Table III.

Work-up 3 and 4 represent a 100% recovery of I and work-up 2 and 4 a 100% recovery of the internal standard. Each work-up was repeated ten times. For each set the mean peak heights were calculated and the percentage recovery of I and II was determined by comparing samples 1/2 and 3/4 and samples 1/3 and 2/4, respectively.

TABLE I

PLASMA DATA FOR PRECISION AND ACCURACY FROM A 50 μg/ml POOL OF I

Sample volume, 50 μl. Sample diluted with 1 ml of control plasma.

Work-up	Concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)
1	50.08 ± 1.53	3.05
2	49.04 ± 1.92	3.92
3	45.09 ± 1.50	3.33
4	56.41 ± 1.66	2.94
5	49.52 ± 1.61	3.25
6	50.78 ± 1.82	3.58
Mean	50.15 ± 3.73	7.43

The recovery from plasma samples was 48.1% for I and 61.5% for the internal standard. The recovery from water-diluted plasma samples was 93.1% for I and 93.8% for the internal standard.

Detection limit

Ten individual plasma standards in the lower range of the calibration graph were assayed and the values of the mean and standard deviation were calculated. The limit of detection (DL) was obtained according to the following equation: $DL = X + (3sn + 3sn - 1 + \ldots + 3s)/n$, where X is the mean of the blank signal (if any), sn is the standard deviation of an individual concentration range assayed and n is the number of concentration ranges assayed. The detection limit was

TABLE II

PLASMA DATA FOR PRECISION AND ACCURACY FROM A 5 μg/ml POOL OF I

Sample volume, 50 μl. Sample diluted with 1 ml of control plasma.

Work-up	Concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)
1	4.60 ± 0.23	5.05
2	4.45 ± 0.30	6.79
3	4.91 ± 0.25	5.10
4	5.11 ± 0.46	8.90
5	4.56 ± 0.45	9.94
6	4.74 ± 0.40	8.35
Mean	4.73 ± 0.40	8.51

TABLE III

PROCEDURE USED FOR RECOVERY STUDY

	Work-up 1	Work-up 2	Work-up 3	Work-up 4
(A)	1000 μl plasma	1000 μl plasma	1000 μl plasma	1000 μl plasma
(B)	50 μl plasma	50 μl plasma	50 μl plasma	50 μl plasma
	1000 μl water	1000 μl water	1000 μl water	1000 μl water
	50 μl I[a]	50 μl I[a]	50 μl acetonitrile	
	50 μl II[b]	50 μl acetonitrile	50 μl II[b]	100 μl acetonitrile
	↓	↓	↓	↓
	Add diethyl ether and concentrated hydrochloric acid, shake, centrifuge and transfer as much diethyl ether as possible. Evaporate to dryness			
	↓	↓	↓	↓
	100 μl acetonitrile	50 μl acetonitrile	50 μl I[a]	50 μl I[a]
		50 μl II[b]	50 μl acetonitrile	50 μl II[b]
	↓	↓	↓	↓
	Evaporate to dryness and dissolve the residue in ethyl acetate. Transfer into a sample vial, evaporate to dryness and derivatize. Evaporate to dryness and take up the residue in (A) 50 μl and (B) 100 μl acetonitrile. Inject 1 μl on to gas chromatograph			

[a] Acetonitrilic standard solution, 2.0 μg/ml.

[b] Acetonitrilic internal standard solution, 10 μg/ml.

TABLE IV

DETECTION LIMIT OF I IN HUMAN PLASMA

Sample volume (μl)	Concentration range assayed (μg/ml)	Detection limit (μg/ml)	Calibration range (μg/ml)
1000	0.04/0.08/0.15/0.3	0.02	0–0.6
50[a]	0.3/0.5	0.06	0–0.8
50[b]	0.3/0.5	0.03	0–6.5

[a] Diluted with 1 ml of drug-free control plasma.

[b] Diluted with 1 ml of deionized water.

determined for different sample volumes and the influence of the sample matrix was also proved. Table IV shows the results of these measurements.

CONCLUSION

This paper reports a sensitive and selective GC method with electron-capture detection for the determination of a new blood glucose-lowering agent in plasma

(serum) and urine samples. Diethyl ether was found to be an efficient solvent for single-step plasma (urine) extraction, providing low-level drug quantitation without any interference from biological fluid constituents. The assay allows the analysis of the large number of samples required for pharmacokinetic studies; up to 100 samples per day can be analysed. The method is easy to use for routine analysis and has been used successfully for thousands of samples.

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