Journal of Chromatography, 228 (1982) 340—345
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1141

Note

Extractive alkylation of probenecid in plasma and cerebrospinal fluid and determination by electron-capture gas chromatography

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(First received August 14th, 1981; revised manuscript received October 16th, 1981)

Probenecid, 4-(dipropylsulfamoyl)-benzoic acid, is clinically used as an uricosuric agent in the treatment of gout. The drug has also been used to inhibit the renal excretion of penicillins.

The pharmacological action of probenecid is largely confined to inhibiting the renal tubular transport of organic acids. In higher doses than are required for the uricosuric effect, probenecid also inhibits the transport of organic acids at other locations; e.g. the transport system that removes acid metabolites of transmittors from the cerebrospinal fluid (CSF) [1, 2]. This finding led to the introduction of the "probenecid test" for evaluation of the in vivo turnover rate in man by measurement of the probenecid-induced accumulation of acid metabolites in lumbar cerebrospinal fluid [3, 4].

Several problems exist in the assessment of the degree of blockade produced by probenecid. Recently, it was established that CSF concentrations of probenecid metabolites as well as their inhibitory activity were low, and they thus contributed only to a very minor extent to the blockade of acid transport out of the CSF induced by probenecid [5]. Another problem, which has to be settled for optimal use of the "probenecid test", is a proper knowledge of the pharmacokinetics of the drug in man. For this, a highly selective, sensitive and reliable analytical method is required.

Probenecid has been determined by spectrophotometric methods [6, 7], and by gas chromatography with flame ionization [8] or mass spectrometric detec-

0378-4347/82/0000—0000/$02.75

tion [9]. A disadvantage of these methods is the relative insensitivity, requiring large sample volumes when analyzing probenecid in biological fluids. Probenecid has also been determined by electron-capture gas chromatography after esterification with pentafluoropropanol [10]. Very recently a sensitive and selective method for the determination of small plasma volumes by high-performance liquid chromatography was published [11].

This study evaluates a selective and sensitive analytical method for the direct determination of probenecid in small sample volumes (0.1 ml) for pharmacokinetic studies.

EXPERIMENTAL

Apparatus

Gas chromatography. A Pye GCV and a Packard 427 gas chromatographs were used with flame ionization or electron-capture detector operated in the constant-current mode. The glass column (150 × 0.2 cm I.D.) was filled with 5% OV-17 on Gas-Chrom Q, 80—100 mesh, and operated at 210°C. The injector and detector temperatures were 270°C and 290°C, respectively. The flow-rate of nitrogen, used as carrier gas, was 30 ml/min.

Mass spectrometry. The O-methyl derivative of probenecid was identified in an LKB 2021 gas chromatograph—mass spectrometer using an ionization energy of 70 eV. Separation was performed on a 10% SE-30 column with an oven temperature of 250°C.

Reagents and chemicals

Methyl iodide was from E. Merck (Darmstadt, G.F.R.). Tetrabutylammonium hydrogen sulphate (Labkemi, Stockholm, Sweden) was neutralized with 1 *M* sodium hydroxide and washed five times with methylene chloride and twice with heptane before dilution to a 0.1 *M* solution with phosphate buffer (pH 7.0). Silver sulphate was a saturated solution in water. Methylene chloride and cyclohexane were of pro analysi quality and supplied by E. Merck. The N,N-diethyl analogue, and also the N,N-dibutyl analogue of probenecid, could be used as internal standard. The standard was dissolved in phosphate buffer (pH 7.0) and diluted to 50 μg/ml with water.

Methods

Determination of partition ratio as tetrabutylammonium ion pair. The partition experiments were done with equal volumes of methylene chloride and aqueous phosphate buffer (pH 7.0) containing tetrabutylammonium ion. Probenecid was dissolved in the aqueous phase in a concentration of $0.5 \cdot 10^{-3}$ *M*. After equilibration for 30 min and centrifugation, the phases were separated and the concentration of acid as anion in the organic phase was determined by gas chromatography after methylation as described below. The extraction constant was calculated as described by Gustavii and Schill [12].

Evaluation of reaction conditions. The extractive methylation of probenecid was studied in the concentration range 0.5—1.0 μg/ml. Probenecid was dissolved in phosphate buffer (pH 7.0), which also contained tetrabutylammonium ion, 0.1 *M*. An equal volume of methylene chloride with methyl iodide

and hexadocosane, used as marker, was added. After the appropriate time, the reaction was quenched by the addition of 1 ml of 0.5 *M* phosphoric acid. One microlitre of the organic phase was injected into the gas chromatograph equipped with the flame ionization detector. The height ratio of "formed derivative" to the marker was calculated.

Determination of probenecid in plasma and in CSF. To the plasma or the CSF sample (0.1—0.2 ml), 0.1 ml of internal standard solution and 1.0 ml of tetrabutylammonium ion solution in 0.1 *M* in phosphate buffer (pH 7.0) were added. This aqueous phase was shaken for 45 min with 1 ml of methylene chloride with 5% of methyl iodide. An aliquot of the organic phase was transferred to another tube with cyclohexane, 0.1 ml. Methylene chloride and methyl iodide were blown off with a stream of nitrogen and 1 ml of cyclohexane was added. Tetrabutylammonium iodide was removed by a silver sulphate wash for 10 min [13]. One microlitre of the organic phase was injected into the gas chromatograph with the electron-capture detector.

A standard curve was prepared in parallel by treating a known amount of probenecid in plasma according to this procedure.

RESULTS AND DISCUSSION

Extraction of probenecid

Probenecid has been extracted from acidified samples with benzene [8], chloroform [9] and ethyl acetate [10]. The extracts from biological samples will contain acidic and neutral components in high concentration. A purification step is thus mandatory before derivatization and gas chromatographic analysis [8].

Isolation of organic acids from biological samples can also be achieved as ion pairs with, for example, quaternary ammonium ions. The extraction constant of probenecid as tetrabutylammonium ion pair was $K_{ex} = 10^{3.5}$. This means a high extraction selectivity to more-polar organic acids normally present in high concentration in the biological sample [14]. After methylation the yield of the probenecid derivative in the organic phase was quantitative.

Reaction conditions

Besides the methylation reaction, several interfering side-reactions may take place in the extractive alkylation. Hydrolysis of the reagent may occur with a high concentration of hydroxide ion in the organic phase, this effect being most pronounced using lipophilic counter-ions at high pH [15]. Buffer anions have also been shown to consume reagent [15].

In the present method, it was possible to use pH 7.0 and tetrabutylammonium ion for a minimum consumption of reagent. The time-dependence of the extractive methylation with different concentrations of methyl iodide is shown in Fig. 1. It could be shown that the reaction rate was proportional to the reagent concentration indicating a pseudo-first-order reaction. In the method, 5% of methyl iodide was used with a reaction time of 45 min.

Previously the metabolites of probenecid were determined in plasma and CSF after extractive methylation [5]. In that case, it was necessary to use tetrapentylammonium ion at pH 10 for quantitative derivatization.

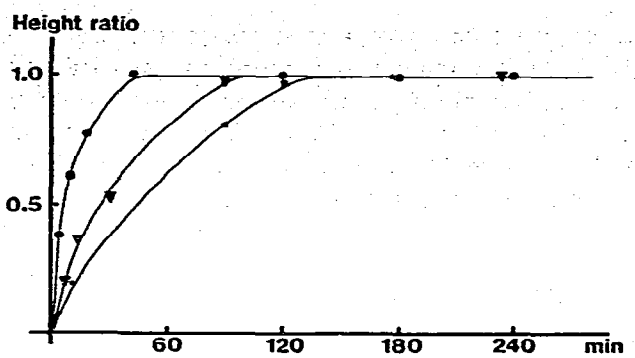

Fig. 1. Extractive methylation of probenecid. Aqueous phase: tetrabutylammonium ion in phosphate buffer (pH 7.0), 1.0 ml. Organic phase: methylene chloride with methyl iodide, 1.0 ml. (⋆ — ⋆) = 1%, (▼ — ▼) = 2% and (● — ●) = 5% of reagent. Concentration of probenecid: $5 \cdot 10^{-3}$ *M*.

Identity of derivative

The derivative of probenecid after extractive methylation was identified by mass spectrometric analysis. The following prominent peaks were seen at *m/e* (percentage relative abundance): 135 (54), 199 (62), 228 (32), 270 (100) and 299 (M^+, *m/e* 30). This confirms the spectra previously shown [8, 9].

Electron-capture response of the derivative

The electron-capture response of a series of sulphonamides with different substituents has recently been presented [15, 16]. The sulphonamide moiety was shown to have a good electron-capturing ability which was strongly amplified by conjugation with carbonyl or phenyl groups. Most sulphonamides, used as antibacterial agents, showed a minimum detectable concentration after methylation in the range $2 \cdot 10^{-16}$ to $10 \cdot 10^{-16}$ moles/sec. Probenecid as its O-methyl derivative has a minimum detectable concentration of $2.5 \cdot 10^{-16}$ moles/sec, corresponding to 3 pg in an injected sample on a column with 1600 theoretical plates and with a retention time of 3 min. The electron-capture response has to be confined to the sulphonamide group adjacent to the phenyl group. Probenecid has previously been determined by electron-capture gas chromatography after esterification with pentafluoropropanol [10]. The contribution from the pentafluoropropyl group to the response must be small in this case. The derivative could easily be detected in amounts as low as 100 pg.

Choice of internal standard

Initially in these studies, the O-ethyl derivative of probenecid was synthesized and used as internal standard. Although a low pH was used in the method, hydrolysis of the internal standard took place. The product was immediately methylated and interfered in the determination. Therefore, the N,N-diethyl or the N,N-dibutyl analogue of probenecid was used as they were extracted similarly and formed the corresponding derivative.

Analysis of probenecid in plasma and CSF

A high selectivity of the method was attained by the extraction conditions used. The derivative of probenecid also showed a high selective electron-cap-

ture response. Owing to these facts, the extractive alkylation could be performed directly in the biological sample saving time-consuming purification steps. Excess reagent was evaporated and tetrabutylammonium iodide formed in the reaction was removed by the silver sulphate wash. No components from the biological sample interfered in the chromatogram. Two chromatograms of an identical plasma sample and of a blank plasma sample are shown in Fig. 2.

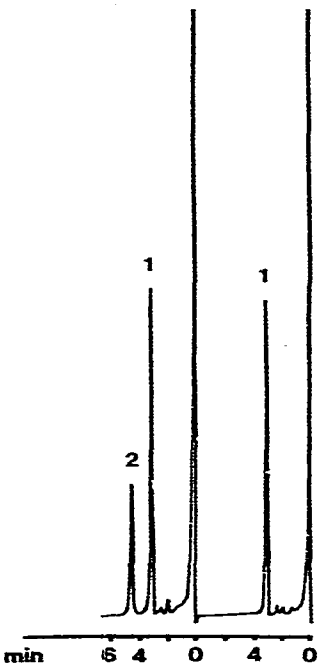

Fig. 2. Chromatogram of a plasma sample with probenecid (2; 2.2 μg in 0.1 ml of plasma) and internal standard (1; 40 μg/ml) run according to the method (left panel), and of a blank plasma sample containing only internal standard (right panel).

The method has been used in pharmacokinetic studies in the quantitation of probenecid in plasma or CSF samples. Linear standard curves through the origin were obtained in the concentration range 1—10 μg/ml probenecid. The lowest amount of probenecid detected was 50 ng in a 0.1-ml plasma sample and the lowest concentration determined was 1 μg/ml, the relative standard deviation at this level being 8.0% (n = 10). The recovery from plasma as compared to a known amount of O-methylprobenecid was 99%.

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