Biochemical Pharmacology, Vol. 17, pp. 1735–1738. Pergamon Press. 1968. Printed in Great Britain

# SHORT COMMUNICATIONS

## Identification by mass spectrometry of pyroglutamic acid as a peak in the gas chromatography of human urine

(*Received* 21 *March* 1968; *accepted* 25 *April* 1968)

WITH the aim of studying histamine metabolism in man the authors have previously developed a gas chromatographic method for the analysis of urinary imidazolic acids.[1–3] The acids were group separated from other components of the urine by ion exchange technique. A strongly basic ion exchange resin was used and in addition to the imidazolic acids, many other carboxylic acids were retained by the column and eluted in the same fractions. After esterification and extraction the pooled fractions were analysed by GLC. In the chromatograms several peaks appeared, some of which could be identified as representing imidazolic acids. In studying the urinary excretion of histamine metabolites by patients with different diseases some observations were also made concerning the gas chromatographic peaks, representing non-imidazolic acids. It was especially evident that one of the peaks, always appearing in the chromatograms of healthy man, was unusually high in the chromatograms of patients with burns and some allergic diseases (Fig. 1). The purpose of the present investigation is to identify the compound representing this high peak.

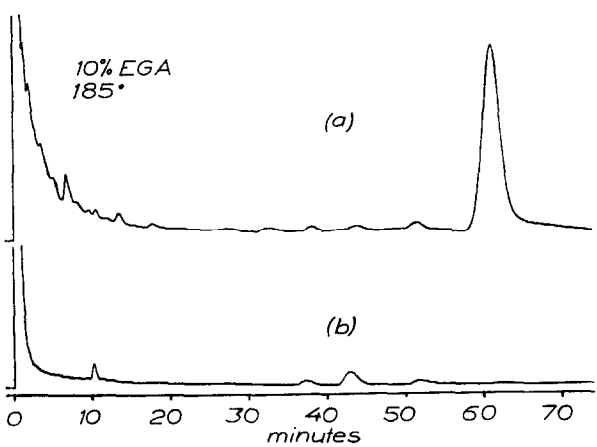


FIG. 1. Gas chromatography of urine extracts, treated as described in text. (a) Urine from a patient with a severe burn. The high peak represents the unknown compound referred to in text. The small peaks represent different imidazolic acids. The size of these peaks correspond to a 24-hr excretion of 2–4 mg. (b) Urine from a healthy man.

## EXPERIMENTAL

*Group separation of the unknown compound from urine and preparation of the methyl esters.* The unknown compound was partly separated from other compounds of the urine, esterified and extracted with chloroform as described in ref. 3.

*Gas chromatographic analysis.* Gas chromatographic analysis was performed as described in ref. 3.

*Isolation of the unknown compound by gas chromatography.* The F & M gas chromatograph, used for the experiments, is equipped with a splitter system at the outlet side of the column. The splitter divides the gas stream into two parts, one of which passes to the detector and the other to a port into which a teflon tube can be inserted.

The first column to be used was packed with 10% EGA on Gas-Chrom P, 100–120 mesh. The column was 1·8 m × 7·2 mm i.d. U-formed glass tube, especially prepared for this semi-preparative use. It was operated at 180°. A urine extract was injected in the gas chromatograph and when the "unknown compound" began to appear in the chromatogram a teflon tube (0·4 m) was inserted into the splitter port and the compound condensed on its walls. The procedure was repeated several times; the same teflon tube was used without rinsing. When a sufficient amount of the unknown compound had condensed, methanol was allowed to drop through the tube in order to elute the substance. The volume of the methanolic solution was reduced *in vacuo*.

The EGA column was then exchanged for a column, packed with 5% SE-30 on Gas-Chrom P, 2·5 m × 3·2 mm i.d., operated at 140°. Samples of the methanolic solution were injected and the unknown compound was again collected at the outlet side of the column as described above.

*Gas chromatography—mass spectrometry.* The technique, described by Ryhage was used.[4]

*High resolution mass spectrometry.* Atlas equipment was used.

*Thin layer chromatography* (TLC). The plates were coated with a layer, 300 μm thick of Merck Silica gel G and dried at 80° for 1 hr. Solvent system: Aceton + ethanol + distilled water (50:10:3).

## RESULTS AND DISCUSSION

A urine extract from a patient with a severe burn was prepared and esterified as described previously. Gas chromatography on an EGA-column revealed large amounts of the "unknown compound". The first clue to the identity of the substance was obtained by analysis of a sample of the urine extract in the combined gas chromatograph—mass spectrometer.[4] A mass spectrum recorded from the gas chromatographic effluent corresponding to the unknown peak revealed a molecular ion peak at *m/e* 143, a base peak at *m/e* 84 (M-59; 59 = $COOCH_3$) and other peaks at *m/e* 85, *m/e* 56 and *m/e* 41. In order to get further information about the substance small amounts were isolated by gas chromatography. The compound was collected from the effluent of an EGA-column in a coiled teflon tube as described under Experimental. About 0·7 mg of the unknown compound could be collected each time. Urine extracts were injected 10 times yielding a total of about 7 mg isolated material. Unfortunately, the material was contaminated by "bleeding" from the column polyester phase. It was therefore necessary to run the isolated material through another column with less bleeding. With an SE-30 silicon phase the unknown compound appeared in the chromatogram as a narrow peak with a short retention time (about 5 minutes at 140°). The isolated material was run through this column in 5 different injections. A total of 3 mg pure substance was collected from the effluent corresponding to the peak. The compound condensed as a white powder in the teflon tube and was dissolved in a small amount of methanol. When 100 μg was spotted on a thin layer plate it could be stained with ninhydrin, indicating a primary or secondary amino group. About 1 mg of the substance was introduced in a high resolution mass spectrometer and a "peak matching" of the molecular ion peak (*m/e* = 143) was carried out. This gave the empirical formula

$$C_6N\,O_3\,H_9$$

With the accumulated information it could be suggested that the identity of the unknown substance was *pyroglutamic acid methyl ester*.

Authentic pyroglutamic acid (Calbiochem) was esterified with HCl-methanol and the gas chromatographic properties of the compound were established. The retention time of authentic pyroglutamic acid (methyl ester) on two columns (EGA and SE-30) was in agreement with the retention time of the unknown peak of the urine extract. When run on a thin layer plate the $R_f$ values of authentic methyl ester of pyroglutamic acid and of the compound isolated by gas chromatography from human urine were the same.

A mass spectrum recorded from the effluent corresponding to the unknown peak of the urine chromatogram showed close resemblance to the mass spectrum of the methyl ester of authentic pyroglutamic acid (Fig. 2).

Thus, the unknown compound in the urine extract was proven to be the methyl ester of pyroglutamic acid. The physiological significance of this observation requires further study. The formation of pyroglutamic acid from glutamic acid has been proven to be an enzymic reaction in certain mammals.[5-7] However, it is also known that glutamic acid can be nonenzymically converted to

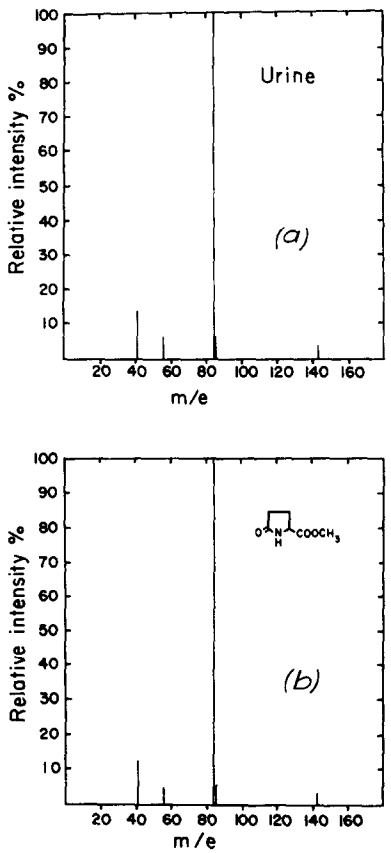


FIG. 2. (a) Mass spectrum of the unknown gas chromatographic peak in urine extracts from a patient with a severe burn. (b) Mass spectrum of the methyl ester of authentic pyroglutamic acid.

pyroglutamic acid. Available data indicate that glutamic acid in the urine may be converted to pyroglutamic acid during the separation and esterification procedure. It is thus not known if the high peak in the gas chromatograms of urine extracts from patients suffering from burns and some allergic diseases corresponds to a high excretion of glutamic acid or/and pyroglutamic acid.

A method for the gas chromatographic determination of pyroglutamic acid has previously been described by Polgar and Meister.[8]

This work was supported by grants from the National Institutes of Health, U.S.A. (GM 13978, Holmstedt-Sjöqvist) and from the Swedish Medical Research Council (40X-677-01, Holmstedt-Tham).

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Biochemical Pharmacology, Vol. 17, pp. 1738–1741. Pergamon Press. 1968. Printed in Great Britain

# Ageing and reactivation of acetylcholinesterase inhibited with Soman and its thiocholine-like analogue

(*Received* 27 *February* 1968; *accepted* 25 *April* 1968)

THE ANTIDOTAL effect of oximes in combination with atropine is negligible in *O*-pinacolyl methylphosphonofluoridate (Soman) poisoning.[1, 2] According to Loomis and Johnson[3] this is due to rapid ageing of the Soman inhibited acetylcholinesterase (AChE). Experiments *in vitro* seem to support this assumption.[3, 4] However, studies of ageing in the brain of the rat *in vivo* (measured by the rate of dealkylation) have shown that maximum dealkylation was reached only 30 min after poisoning with $^{32}P$ Soman.[5]

It follows from this, and from other results *in vivo*,[6, 7] that in spite of ageing, there might be enough time for an oxime to reactivate effectively Soman inhibited AChE and restore function at cholinergic synapses. Since treatment fails even if the oxime is given before Soman,[1, 2] some other factors besides ageing have been considered. One of them was that bulky alkoxy side chain of Soman hinders the binding of oxime to the anionic site of the enzyme (Heilbronn and Tolagen[2]). Alternatively the toxicity of TMB-4 could limit the dose of oxime required for an effective concentration *in vivo* (Berry *et al.*[8]).

To check the hypothesis that the alkoxy side chain of soman limits the effectiveness of oximes, we studied the reactivation of AChE, ageing and protective effect of 1,3-trimethylenebis(4-hydroxyiminomethylpyridinium chloride) (TMB-4) in poisoning by Soman and its thiocholine-like analogue (compound I). Compound I differed from Soman in containing a -S-(2-diethylaminoethyl) methylsulphomethylate group instead of fluorine.

## EXPERIMENTAL AND RESULTS

One volume of washed human haemolysed erythrocytes was mixed at 0° with 1 vol. of a cold aqueous solution of the organophosphorus compounds. The final concentration of the organophosphates was $10^{-7}$ M and inhibition of the enzyme amounted to about 90 per cent; 15 min later 0·08 ml of the mixture was transferred to a vessel kept in a water bath at the 37° and containing either acetylcholine chloride (ACh) or ACh plus TMB-4 and the activity of AChE was determined by a modification of Michel's electrometric method.[9] The percentage reactivation of AChE (Fig. 1) was calculated as suggested by Hobbiger.[10]