Journal of Chromatography, 290 (1984) 303–310
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 265

APPLICATION OF GAS CHROMATOGRAPHY–MASS SPECTROMETRY TO THE STUDY OF BIOPTERIN METABOLISM IN MAN

DETECTION OF BIOLUMAZINE AND 2'-DEOXYSEPIALUMAZINE*

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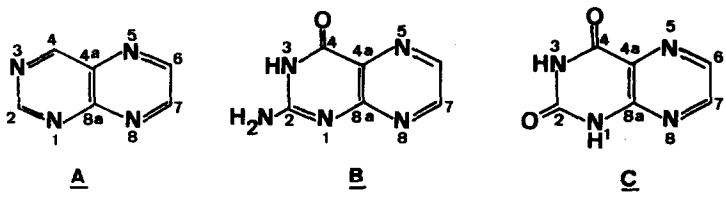
SUMMARY

The separation characteristics of the trimethylsilyl ether derivatives of various naturally occurring and synthetic pteridines on a apolar glass capillary column, together with their mass spectra, permit their identification and quantitation in biological samples. Examples are given of the determination of the ratio of monapterin to neopterin in urine, of monitoring excreted pterin metabolites after a loading test with 6-methyltetrahydropterin in urine and of structure elucidation of lumazines, previously unknown in man. 6-Methylisoxanthopterin was shown to be the main metabolite in urine after administration of 6-methyl-5,6,7,8-tetrahydropterin. Biolumazine and 2'-deoxysepialumazine were found in human faeces after administration of (6*R*,*S*)-5,6,7,8-tetrahydro-L-*erythro*-biopterin.

INTRODUCTION

Pteridines, the pigments of the butterfly wing, were already known as substance class at the end of the last century, but their structures were elucidated only in the 1940s when the basic heterocycle was recognized to have the pteridine structure, shown in Scheme 1, A.

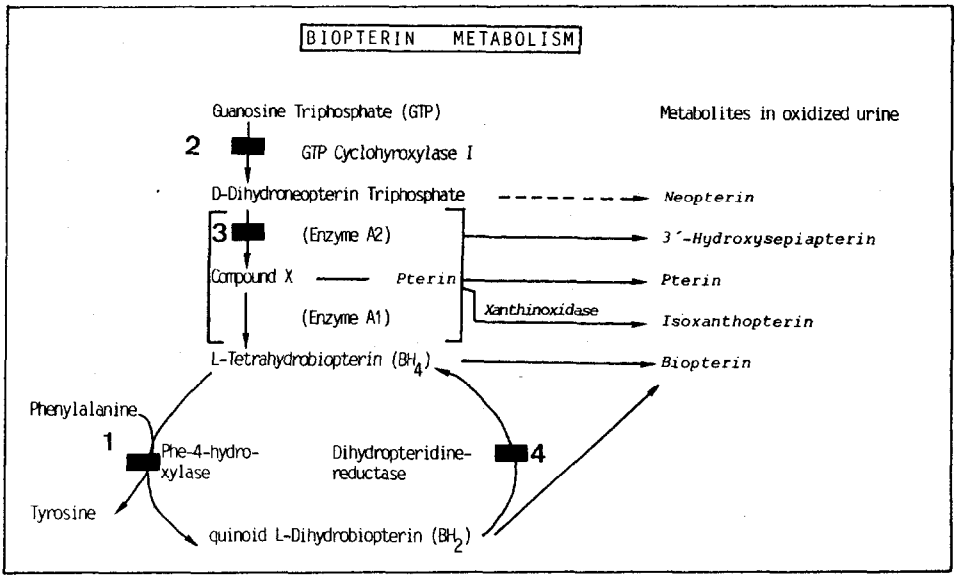


Scheme 1. Structural formulae of some pteridines. (A) Pteridine; (B) 2-amino-4-hydroxypteridine (pterin); (C) 2,4-dihydroxypteridine (lumazine).

* Dedicated to Prof. Dr. H.-Ch. Curtius on the occasion of his 60th birthday.

0021-9673/84/$03.00

According to IUPAC[1], nowadays the term pterin, earlier used as a collective name, represents only for the 2-amino-4-hydroxypteridine residue (Scheme 1, B). During the 1960s, pteridines, especially those substituted in position 6, gained increasing interest in biochemistry and medicine, when Kaufman[2] and others discovered that biopterin is the coenzyme for different monooxygenases. It has been shown more recently that the group of diseases known as phenylketonuria (PKU) includes some rare atypical forms that are caused by defects in the tetrahydrobiopterin (BH_4) metabolism[3–5]. BH_4 here is the cofactor of the monooxygenases: phenylalanine-4-hydroxylase, tyrosine-3-hydroxylase and probably tryptophan-5-hydroxylase, which are the key enzymes in the synthesis of different biogenic amine neurotransmitters (see ref. 6 for an overview).



Scheme 2. Biopterin metabolism and pterins analysed in urine. Blocks in the biosynthesis: 1 = phenylketonuria (PKU); 2 = GTP cyclohydrolase I deficiency; 3 = BH_2 synthetase deficiency; 4 = dihydropteridine reductase deficiency.

Scheme 2 shows the biopterin metabolism, which contains several steps that are not yet understood[6], and the metabolites that can be found in urine. The numbers in Scheme 2 denote possible blocks in the metabolic pathway, namely (1) classical PKU, (2) GTP cyclohydrolase I deficiency, (3) BH_2 synthetase deficiency and (4) dihydropteridine reductase deficiency. It is evident from Scheme 2 that the analysis of the urine metabolites, especially the ratio of biopterin to neopterin, can be used to localize defects in the metabolic pathway. This analysis can be done routinely by high-performance liquid chromatography (HPLC)[7], which is highly sensitive and easy to perform without extensive sample pre-purification.

In some instances, especially for metabolic profiling, structure elucidation of unknown pteridines or confirmation of other analytical methods, gas chromatography–mass spectrometry (GC–MS) as a sensitive and selective method is

undoubtedly the method of choice. Some examples from our laboratory may serve as an illustration.

EXPERIMENTAL

Cleanup

Urine (1 ml) was oxidized with manganese dioxide (pH 1.5) and placed on a Florisil (H^+) column. After washing with 10 ml each of 0.1 *M* hydrochloric acid and water, pteridines were eluted with 10 ml of acetonitrile–pyridine–ammonia–water (60:3:4:40) and the eluate was diluted with 10 ml of water. The solution was adjusted to pH 10 with 0.1 *M* sodium hydroxide solution and subjected to chromatography on a Lewatite MP 5080 (Ac^-) column. After washing with 20 ml of water, pteridines were eluted with 10 ml of 0.1 *M* formic acid and the eluate was evaporated to dryness.

Trimethylsilylation was performed with a 300 μl of a 1:1 (v/v) mixture of bis-trimethylsilyltrifluoroacetamide (BSTFA) (Regis, Morton Grove, IL, U.S.A.) and acetonitrile (Fluka, Buchs, Switzerland) at 100°C for 1 h and a 1-μl aliquot of this solution was used for injection into the GC–MS system.

GC–MS

A Carlo Erba Fractovap 2900 chromatograph with a Grob-type split–splitless injector and a 20 m × 0.3 mm I.D. SE-52 glass capillary column (Jaeggi, Trogen, Switzerland) was used. Helium at an inlet pressure of 1.2 bar served as the carrier gas and the injection temperature was 275°C. The temperature programme was 3 min at 180°C, then increased to 250°C at 4°C/min.

The gas chromatograph was coupled to a mass spectrometer via an open split and direct coupling interface[8]. The mass spectrometer was a VG-16F single-focusing magnetic field instrument, directed by a Finnigan Incos 2000 data system, with electron-impact ionization at 40 eV and an ion source temperature of 200°C. The magnet cycle time was 2 sec in the range *m/z* 100–750 and the accelerating voltage was 4000 V.

RESULTS AND DISCUSSION

GC–MS as control for HPLC

During HPLC screening of pterins, the ratio of the amount of L-*threo*-neopterin (monapterin) excreted to that of D-*erythro*-neopterin (neopterin) sometimes exceeds the usual value, which is about 0.1. Fig. 1 shows the HPLC traces of two urine samples with measured monapterin to neopterin ratios of 0.1 (A) and 0.21 (B) (for further chromatographic details, see ref. 7). The question arises of whether the peak observed in the latter instance is really pure monapterin.

Fig. 2 shows the total ion current (TIC) chromatogram of sample B together with a fragmentogram of mass 409 which is the base peak in the mass spectra of biopterin, monapterin and neopterin. Whereas the three compounds are not easily recognized in the TIC trace, monitoring the base peak permits a much clearer identification.

Fig. 3 shows the mass spectra of the two isomeric neopterins, which, together with their methylene units[9], lead to the conclusion that the HPLC peak is indeed

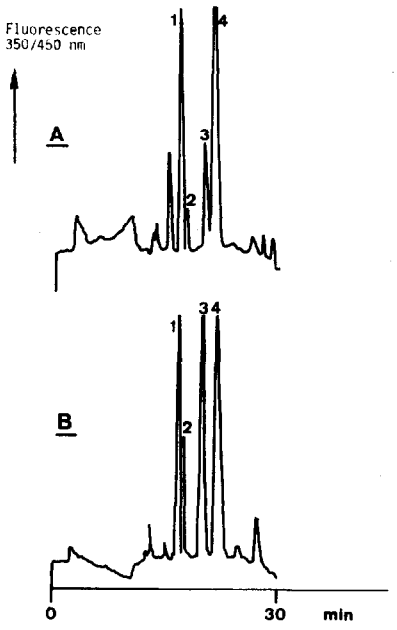


Fig. 1. Reversed-phase HPLC of blue-fluorescing pterins of a urine with (A) a normal and (B) a high monapterin to neopterin ratio. For experimental details, see ref. 7. 1 = Neopterin; 2 = monapterin; 3 = isoxanthopterin; 4 = biopterin.

monapterin. Mass fragmentography of the peak of *m/z* 409 (with the aid of a calibration graph) allows quantitation of the monapterin to neopterin ratio, and MS may therefore serve as a reference method for HPLC.

Fig. 4 shows the calibration graphs for HPLC (broken line) and GC–MS (full line). The arrows mark the respective values for urine sample B (see Fig. 1). HPLC determination yields 0.21 for the above ratio, and the value found by GC–MS is 0.22, which shows good agreement between the two methods.

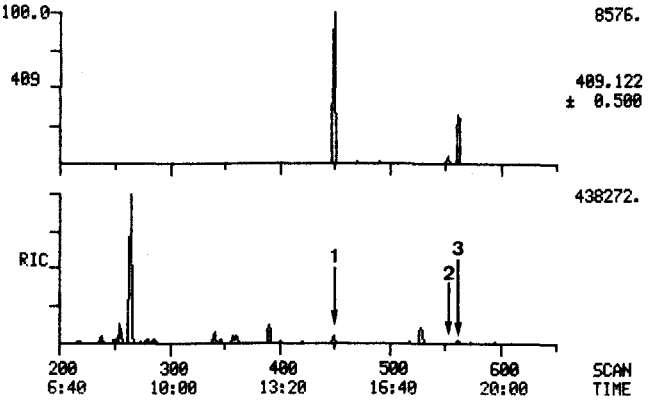


Fig. 2. Mass fragmentogram (*m/z* 409) and TIC chromatogram of urine sample B from Fig. 1. 1 = Biopterin; 2 = monapterin; 3 = neopterin.

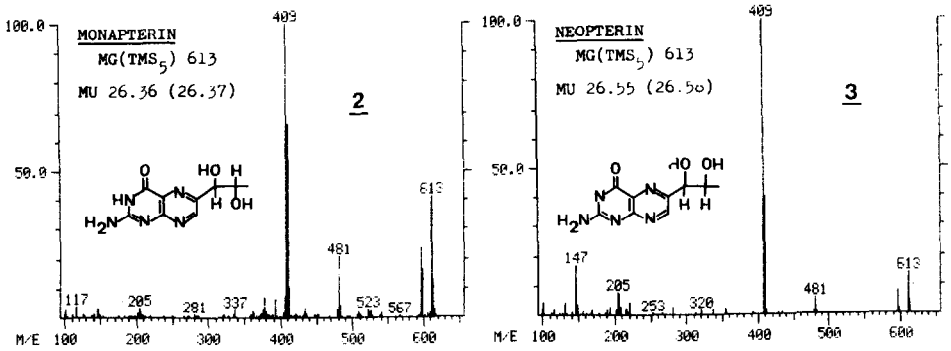


Fig. 3. Mass spectra and methylene units (MU) of L-*threo*-monapterin (2) and D-*erythro*-neopterin (3). MU values in parentheses are reference values.

GC–MS in loading experiments

Patients suffering from atypical forms of PKU can be treated successfully with doses of BH_4. On the other hand, this cofactor only slightly passes the blood–brain barrier which would be essential for the neurotransmitter synthesis. With the aim of finding cofactor substitutes that enter the brain more easily than BH_4, we treated a patient orally with 6-methyltetrahydropterin. Before and after treatment we observed the excreted pterins (2–4, 4–6 and 12–24 h). Before loading, the only identified pterins were biopterin and neopterin, but 6 h later the dominating pterin metabolites were 6-methylpterin and 6-methylisoxanthopterin, which were confirmed by their mass-spectra and methylene units[9]. The TIC chromatogram is shown in Fig. 5.

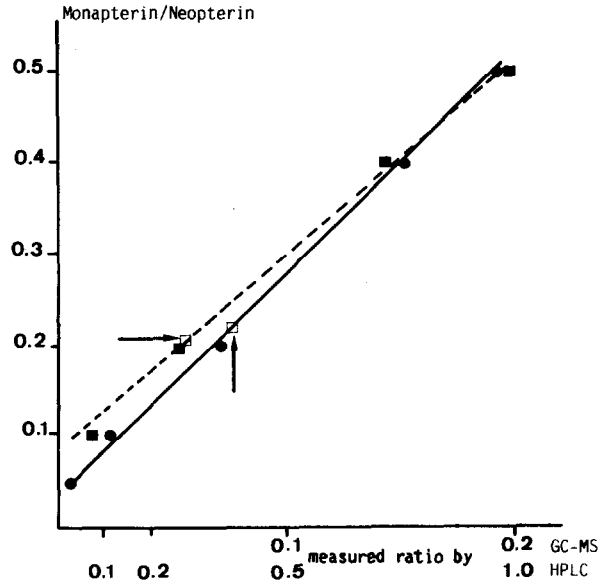


Fig. 4. Calibration graphs and ratio determination for peak areas (arrows) of monapterin to neopterin by HPLC (■ - - - ■) and GC–MS (●——●). Ratio values determined, (□) using the corresponding calibration line: HPLC, 0.21; GC–MS, 0.22.

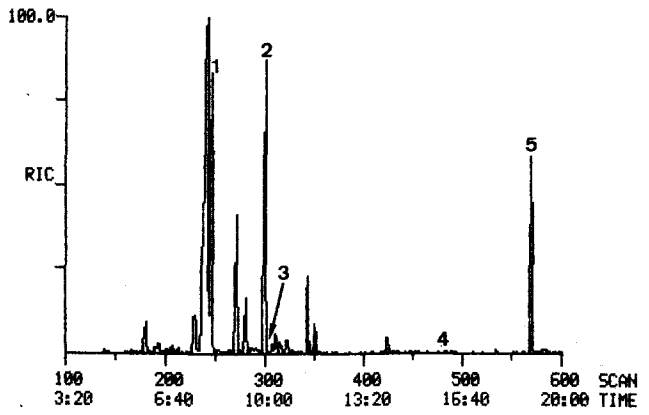


Fig. 5. TIC chromatogram of a urine sample 4–6 h after loading with 6-methyltetrahydropterin. Peaks: 1 = 6-methylpterin (TMS_3); 2 = 6-methylisoxanthopterin (TMS_3); 3 = 6-methylpterin (TMS_4); 4 = biopterin; 5 = neopterin.

The excretion pattern in Fig. 6 shows a maximum between 2 and 4 h after loading. There is a significantly slower decrease of 6-methylisoxanthopterin (from 25 to 0.7) than 6-methylpterin (from 212 to 0.3) in Fig. 6. This is possibly due to the fact that the measured 6-methylpterin originates from 6-methyltetrahydropterin, which wasexcreted mainly unchanged, whereas 6-methylisoxanthopterin is produced by the action of xanthine oxidase.

GC–MS for structure elucidation of new metabolites

When patients are loaded with BH_4, the recovery of this compound or its metabolites is only 1–5%. The question therefore arises as to whether some pterins

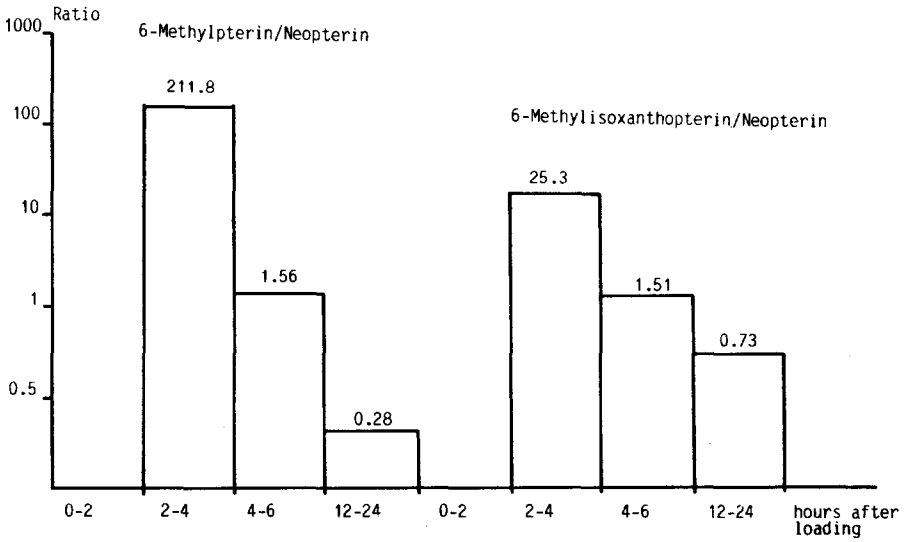


Fig. 6. TIC peak-area ratios of 6-methylpterin to neopterin and 6-methylisoxanthopterin to neopterin *versus* time after loading with 6-methyltetrahydropterin.

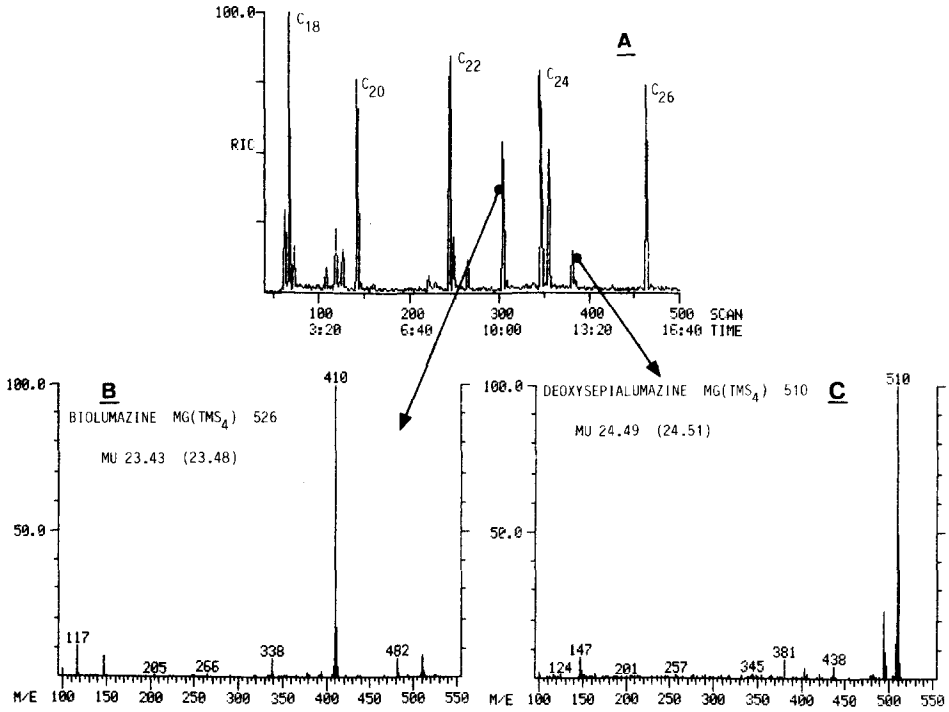


Fig. 7. TIC chromatogram of a faeces sample (spiked with even-numbered *n*-alkanes (C_{18}–C_{26}) and the mass spectra of two identified lumazines together with the determined methylene units. Values in parentheses originate from reference compounds.

are also excreted in the faeces. Fig. 7 shows the TIC chromatogram of such a sample together with the mass spectra of two identified compounds (Fig. 7B and C). In order to determine the methylene units, the sample was spiked with even-numbered *n*-alkanes (C_{18}–C_{26}).

Owing to the characteristic fragmentation of tetrahydro-, dihydro- and aromatic pteridines and the fact that the even-numbered molecular weight of the trimethylsilyl derivatives is indicative of a lumazine skeleton[9], the structures shown in Fig. 7B and C could be proposed. By running reference samples under identical conditions and comparing the spectra and the methylene units, the identification of biolumazine and 2′-deoxysepialumazine in this faeces sample was confirmed.

Going back to the original question of whether a high percentage of loaded pterins is excreted in the faeces, one must admit that this is not so, as the amount lies in the same range as for urine. However, the experiment led to the detection of some pteridines that were not previously known to occur in humans and, for such questions, GC–MS has advantages over other very sensitive and easy to perform analytical methods.

ACKNOWLEDGEMENT

We thank Professor W. Pfleiderer, University of Constance, F.R.G., for gifts of reference compounds, and Mr. W. Scherrer for skilful technical assistance.

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